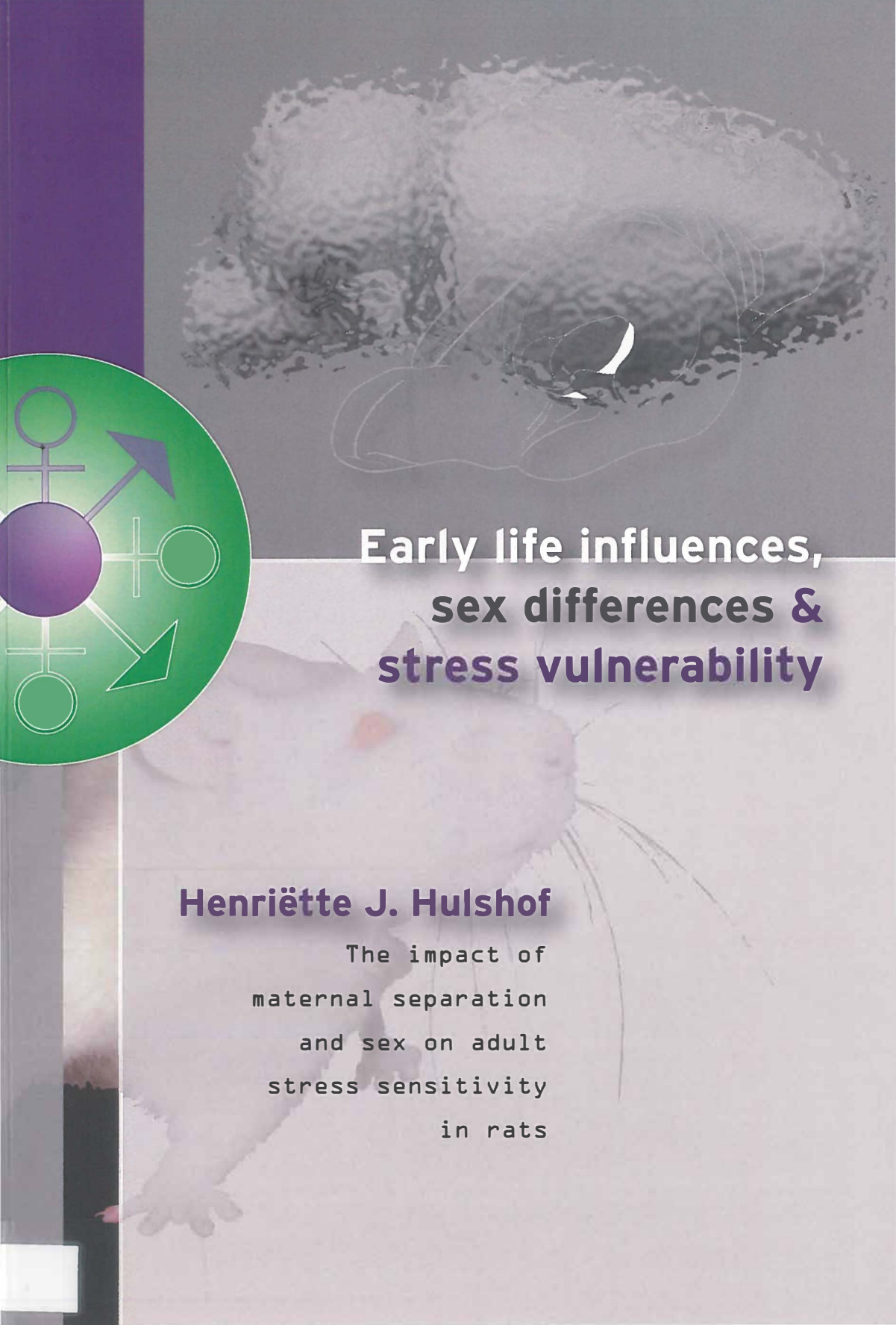
Early life influences, sex differences & stress vulnerability : The impact of material separation and sex on adult stress sensibility in rats

Hulshof, Henriëtte Jolanda



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Henriëtte J. Hulshof

The impact of maternal separation and sex on adult stress sensitivity in rats

Early life influences, sex differences and stress vulnerability

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Stellingen

behorende bij het proefschrift

Early life influences, sex differences and stress vulnerability

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Henriëtte Jolanda Hulshof

1. In tegenstelling tot hetgeen de Engelse uitdrukking "As the twig is bent, the tree's inclined"[1] suggereert, hebben (negatieve) gebeurtenissen vroeg in het leven niet altijd grote gevolgen voor de toekomst. (dit proefschrift)

2. De populaire ideeën over het maternale separatie model geven aan dat foute aannames over algemene principes, gebaseerd op inconsistente data, lang stand kunnen houden. (dit proefschrift)

3. Als promovendus werkend aan stressonderzoek krijg je te maken met het Droste-effect.

4. Naarmate de tijd vordert, blijkt het produceren van een proefschrift in vier jaar alleen een haalbare kaart, wanneer het zuivere speeltijd zou betreffen en daarnaast ook alle spelers in het veld blijven staan.

5. Om de kwaliteit van gepubliceerd onderzoek te waarborgen, zou ook de peer review procedure dubbelblind moeten worden uitgevoerd.

6. Het "publish or perish" principe houdt geen rekening met het feit dat onderzoek doen geen garantie is voor het vinden van significante data, net als vissen niet betekent dat je altijd iets vangt.

7. De analogie tussen wetenschap/promoveren en cricket: zij die het bedrijven, lijken precies te weten wat ze doen; de rest heeft geen idee.

8. The act of putting pen to paper encourages pause for thought [...][2]

9. Zonder muziek zou het leven niet zozeer een vergissing zijn, maar ondenkbaar.[3]

10. De meest gelezen delen van het proefschrift kosten de minste tijd om te schrijven.

[1] Alexander Pope, English poet
[2] Norbert Platt, former CEO Montblanc
[3] "*Ohne musik wäre das leben ein irrtum*", Friedrich Nietzsche, German philosopher & poet

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Ich kann, weil ich will, was ich muss
I. Kant

voor
en
dankzij
mijn lieve
ouders & broer

Contents

1. General introduction

Julian Velard - Joni

1. Background

It has long been recognized that the response to stress is a double-edged sword. While the acute activation of the sympatho-adrenomedullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis is essential for normal stress adaptation, chronic and excessive exposure to high levels of stress hormones appears to be harmful and might predispose individuals to the development of a wide range of physiological and psychological disturbances. Adverse or stressful experiences during early life may be even more detrimental since then stress potentially interferes with the normal brain development, which might result in functional alterations that persist into adulthood. Child abuse and neglect or parental loss were found to be important risk factors for the development of psychopathologies and diseases later in life, including depression, substance abuse, heart disease and cancer (Felitti et al., 1998). The first part of this thesis followed up on previous findings reported in literature and investigated the long-term consequences of early postnatal stress, or more specific maternal separation, particularly how this affects adult stress reactivity.

Apart from early life experiences, sex was found to be another factor to play a prominent role in individual differences in adult stress sensitivity. Epidemiological studies showed a clear sexually biased prevalence of stress-related pathologies, such as depression and sleep complaints. Studying the mechanisms underlying sex differences is rather complex, with genetic influences as well as hormonal modulation by estrogens and androgens. The second part of this thesis focuses on male-female differences in adult stress sensitivity and the possible role of estradiol herein.

The following introduction summarizes research relating to the influence of early life stress and sex on adult stress sensitivity and provides a background for the experimental chapters.

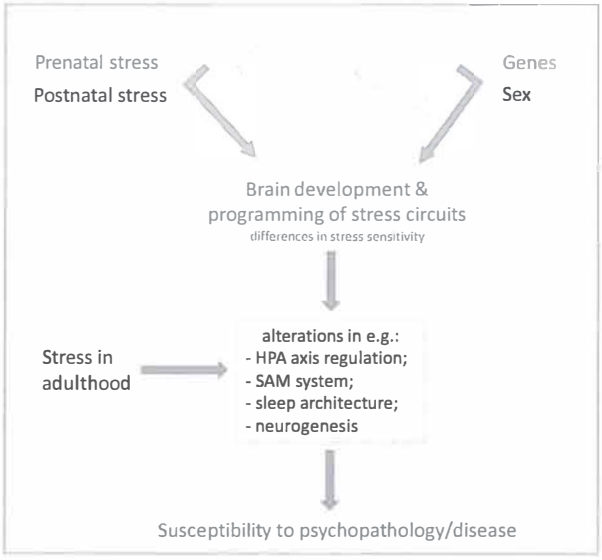


Figure 1 *Factors involved in the individual differences in stress sensitivity and the susceptibility to physiological and psychological disturbances. A genetic predisposition and environmental influences may alter stress systems, neuroplasticity and sleep, which in turn might increase the susceptibility to mood disorders and other diseases. Besides a direct effect of genetic and environmental factors, e.g. neurogenesis might also be influenced by a stress-induced disruption of sleep. The present thesis focuses on the influence of early life stress and sex on differences in stress sensitivity.*

2. Stress

2.1. Stress response systems

The exposure to stress elicits complex endocrine, autonomic and behavioral responses. The main systems that are activated during stress exposure are the hypothalamic-pituitary-adrenal (HPA) axis, the sympatho-adrenomedullary (SAM) and sympathoneural system, which are anatomically and functionally connected (for review, see Chrousos and Gold, 1992). Although the responses of the different stress systems are partly dependent on the type and duration of the stressful experience, generally a stressful stimulus is followed by the rapid, neurally mediated activation of the sympathetic system, while simultaneously suppressing the parasympathetic activity, and a somewhat slower response of the HPA-axis, a neuroendocrine cascade of humorally mediated processes. The activation of these stress systems, but also the subsequent inhibition of the system at the end of the stress exposure, is necessary for adaptation and survival in a continuously changing environment. The limbic system, different nuclei in the medulla oblongata, which is part of the brain stem, and the locus coeruleus (LC) - located in the pons region - play a major role in the initiation of the stress response of both the sympathetic system and the HPA-axis (for a detailed description see reviews by Chrousos and Gold, 1992; Ziegler & Herman, 2002 and Kvetňanský et al., 2009).

The input of stress signals to the medulla oblongata is associated with activation of the sympathoneural system and SAM system. The sympathoneural system activates different organs and tissues through the release of noradrenaline (NA). Activation of the SAM system results in the release of adrenaline (A) from the adrenal medulla. Besides A, the adrenal medulla also produces and releases NA, although in smaller amounts than the ganglionic neurons of the sympathoneural system, which is the major source of circulating NA.

The medulla oblongata also plays a major role in the activation of the HPA-axis. Noradrenergic innervation from the medulla oblongata and LC as well as input from the limbic system towards the paraventricular nucleus (PVN) of the hypothalamus stimulate the release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). The release of CRH into the blood stream leads to the production and release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. ACTH, in turn, stimulates the release of the glucocorticoid corticosterone (CORT) from the adrenal cortex. Glucocorticoids can regulate the activity of the HPA-axis by negative feedback inhibition acting at different levels of the HPA-axis itself, i.e. the hypothalamus and pituitary, but also at the level of central input systems to the HPA-axis, i.e. the prefrontal cortex, amygdala and hippocampus. Two types of corticosteroid receptors have been described in the brain, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), the latter showing a higher affinity for glucocorticoids. While only MRs appear to play a role in the maintenance of the basal activity of the HPA axis, both MRs and GRs are suggested to be involved in the activity of the HPA axis during the circadian peak and in response to stress (Spencer et al., 1998).

The SAM stress system and the HPA-axis were found to interact at different levels in the brain and periphery (for review see e.g. Kvetňanský et al., 1995). As previously mentioned, the stress-induced release of NA in the PVN facilitates the activation of the HPA axis. On the other hand, different hormones of the HPA axis can influence, and might even be essential for, the activity of the SAM system. While CRH and ACTH were found to stimulate catecholamine production and release, glucocorticoids showed inhibitory effects on the sympathoadrenal activity (Fenske et al., 1982; Brown et al., 1985; Brown & Fisher, 1986).

Ultimately, the acute activation of the different stress systems and the release of the catecholamines and glucocorticoids leads to several functional adaptations to increase survival chances, including enhanced alertness, elevated heart rate and blood pressure, altered energy metabolism, and simultaneous suppression of e.g. growth and reproductive function (Chrousos and Gold, 1992).

2.2. Early life influences on the stress systems

Considering that the development of the nervous system continues well after birth, early (stressful) experiences during this vulnerable period might have long lasting effects on functioning in adulthood. Much of what is known about the influence of early life stress on development is based on studies in laboratory rodents. In rats, different components of the stress systems are still in development after birth. Besides the ongoing cellular organization in the hippocampus and pituitary, e.g. the sympathetic innervation of the heart was found to be only fully developed after the first postnatal week in rats (Altman & Bayer, 1990b; Seidler & Slotkin, 1979; Siperstein et al., 1954).

Over 40 years ago, Levine showed that early handling in rats, involving brief, daily separations of mother and pups during the first postnatal weeks permanently altered HPA-axis regulation and reactivity (1967). In general, early handled rats show reduced stress-induced ACTH and CORT levels during adulthood (e.g. Meaney et al., 1989; Viau et al., 1993; Meerlo et al., 1999). Subsequently, many studies investigated the effects of the brief disruption of the mother-infant bond on the different stress response systems (for review, see Meaney et al., 1991). The sympathetic system might also be susceptible to early life influences. Handling reduced sympathetic nervous system activity in the heart, but had no effect on sympathoadrenal activity in response to novelty exposure when compared to nonhandled control animals (Young, 2000). Another study reported similar NA levels in handled and control rats in response to stress, while handling significantly reduced the adrenaline response after novelty exposure (Meerlo et al., 1999). In contrast, McCarty and colleagues found similar basal and footshock-induced NA and A levels in handled and nonhandled rats, indicating no influence of handling on the sympathetic system or sympathoadrenal activity (McCarty et al., 1981).

The effects of early handling are thought to be (at least partly) mediated by changes in maternal behavior. Neonatal handling in rats was found to alter the behavior of the mother towards the offspring, increasing the amount of time spent licking and grooming the pups and on arched-back nursing (e.g. Lee & Williams, 1974; Liu et al., 1997). Naturally occurring high levels of maternal care (high licking and grooming and

arched-back nursing) comparable to the amounts found after handling, resulted in a reduced HPA-axis response to restraint stress in adulthood when compared to low maternal care offspring (low licking and grooming and arched-back nursing), which exhibit maternal care levels comparable to non-handled controls (Liu et al., 1997).

While brief periods of neonatal handling or mother-pup separation result in offspring with lower HPA-axis activity and reactivity, longer periods of maternal separation may have opposite effects (for review, see Lehmann & Feldon, 2000). During handling mother and litter are generally separated for a brief period of up until approx. 15 minutes, which is comparable to naturally occurring bouts of absence (Jans & Leon, 1983). Instead, during maternal separation the mother-infant bond is disrupted for a more prolonged period, varying from 3 to 6h daily during the first postnatal weeks until a 24h separation for one single day. Adult rats submitted to maternal separation for 3h per day during the first postnatal weeks showed increased ACTH and CORT levels in response to stress when compared to nonhandled rats (Aisa et al., 2007; Huot et al., 2002; Plotsky et al., 2005). In agreement with this, maternal separation was found to decrease hippocampal GR density, thereby reducing glucocorticoid feedback sensitivity (Aisa et al., 2007). However, other studies show no differences in adult HPA-axis reactivity or even a reduced activity in maternally separated animals (Plotsky & Meaney, 1993; Slotten et al., 2006; Daniels et al., 2004).

Maternal separation-induced alterations in the sympathetic system have also been reported. Maternal separation significantly decreased stress-induced noradrenaline levels in the hypothalamus and hippocampus without changing basal levels (Daniels et al., 2004; Aisa et al., 2007). Basal plasma levels of both NA and A measured after decapitation were increased in maternally separated adult rats, suggesting enhanced sympathetic activity in maternally separated male rats (Díaz Luján et al., 2008). However, there were no significant differences in baseline heart rate and blood pressure between maternally separated and control animals in adulthood (Loria et al., 2010b). Similarly, early deprivation - a model in which pups are individually separated from the dam, suggested to constitute a more severe early life stressor than maternal separation (Pryce & Feldon, 2003) - in borderline hypertensive rats did not alter basal heart rate and blood pressure, but did increase the restraint stress-induced heart rate response when compared to control rats (Sanders et al., 2007). Likewise, maternal separation might, similar to the effect on the HPA-axis, increase the sensitivity of the sympathetic stress system to subsequent stress exposure. Together, some studies suggest that prolonged maternal separation might have effects opposite to the outcome of brief neonatal handling, with increased HPA-axis and SAM system activity, but the data are less consistent.

2.3. Sex differences in stress reactivity

The development of stress systems and adult stress reactivity may be sex-dependent. In the 1960s it was first recognized that HPA-axis regulation and reactivity is sexually dimorphic. Several early studies showed higher basal and stress-induced CORT levels in female rats as compared to males (Kitay, 1961; Critchlow et al., 1963). Furthermore, females showed higher CRH mRNA levels and lower GR mRNA levels in the PVN than males (Seale et al.,

2004a). Often, these sex differences were attributed to sex hormones. While estrogens have an excitatory role in HPA regulation, androgens appear to have an inhibiting effect on CORT release. Ovariectomy (OVX) was found to decrease basal CORT levels in females, while estradiol administration reversed this OVX-induced decrease (Kitay, 1963; Seale et al., 2004b). Furthermore, OVX reduced ACTH levels and CRH mRNA and increased GR mRNA in the PVN of the hypothalamus (Seale et al., 2004b). Gonadectomy in male rats increased basal and stress-induced CORT levels when compared to sham-lesioned males (Seale et al., 2004a), whereas testosterone replacement returned CORT levels to those found in intact males (Seale et al., 2004b). Gonadectomy in males furthermore increased plasma ACTH levels and PVN CRH mRNA while reducing GR mRNA in the PVN, which was all reversed by testosterone replacement (Seale et al., 2004b). This demonstrates that both male and female gonadal steroids influence HPA axis regulation, which might partly explain the sex differences in HPA (re)activity.

Although several studies replicated the initial findings from Kitay and Critchlow showing a male-female difference in HPA-axis activity (e.g. Kant et al., 1983; Aloisi et al., 1994), the presence or absence of a clear difference may depend on the phase of the estrous cycle. Most studies investigating sex differences in stress reactivity did not distinguish between the different phases of the cycle. Female rats exhibit changing estradiol levels, and correlated changes in CORT levels, during the different phases of the cycle, with peak estrogen and CORT levels during the proestrus phase (Critchlow et al., 1963; Raps et al., 1971; Nequin et al., 1979). While one study showed higher basal CORT levels only in proestrus female rats compared to males (Raps et al., 1971), others reported higher basal CORT levels in diestrus and proestrus females when compared to males (Atkinson & Waddell, 1997). In contrast, Shors and colleagues reported higher basal CORT levels and stress responses in females compared to males, independent of the phase of the female cycle (Shors et al., 1999). Similarly, another study reported higher stress-induced CORT levels in females independent of cycle stage, while a higher ACTH response to footshock was specifically seen in proestrus females when compared to males (Rivier et al., 1999).

Sex differences in the activity of the sympathetic nervous system and the SAM system have also been reported. Basal levels of NA and A were approximately two times higher in females than males (Zukowska-Grojec et al., 1991, Weinstock et al., 1998; Trentani et al., 2003). In contrast, Westenbroek and colleagues reported similar basal A levels in male and female rats (Westenbroek et al., 2005). At least one study observed a significantly higher mean arterial pressure, but not heart rate, in males than estrus or diestrus females under baseline conditions (Weinstock et al., 1998). Others reported similar basal blood pressure and heart rate in male and female rats (Zukowska-Grojec et al., 1991). In response to stress, males showed higher and longer-lasting increases in both blood pressure and heart rate than females (Zukowska-Grojec et al., 1991).

3. Sleep

3.1. Sleep regulation

A commonly reported consequence of stress is disrupted sleep, which in turn might affect cognitive function and mood. The effects of stress on sleep may differ between individuals, depending on various factors including developmental programming and sex.

Sleep occupies about a third of our lifetime and is essential to maintain normal (brain) function. Although the specific function of sleep still has to be elucidated, there are several hypotheses suggesting that sleep may serve a restorative function and play a role in brain plasticity (Benington & Heller, 1995; Frank, 2006; Meerlo et al., 2009). Importantly, sleep consists of two distinct stages, rapid-eye-movement (REM) sleep or paradoxical sleep and non-rapid-eye-movement (NREM) or slow wave sleep (SWS), each of which might have its own function. In agreement with the notion that sleep is crucial for normal brain function and optimal performance, sleep loss or disrupted sleep is associated with cognitive impairment and altered neuroendocrine functioning and is an established risk factor for a wide variety of diseases, including cardiovascular disease and mood disorders (Durmer & Dinges, 2005; Banks & Dinges, 2007; Meerlo et al., 2008; Baglioni et al., 2011).

The daily alternation of sleep and wakefulness is the result of a circadian and a homeostatic process (Achermann and Borbély, 2003). The circadian pacemaker, located in the suprachiasmatic nuclei of the anterior hypothalamus, promotes wakefulness during the active phase and maintains sleep during the resting phase. The homeostatic sleep drive is related to the accumulation of a sleep debt during wakefulness.

The regulation of the sleep-wake cycle is a complex interaction of several brain systems and sleep regulatory substances (for review, see Saper et al., 2001; Saper et al., 2005; Schwartz & Roth, 2008). Arousal or waking is mediated by the activation of ascending pathways projecting from the reticular formation in the upper brain stem towards the thalamus, hypothalamus and basal forebrain, which all project to the cerebral cortex. It is suggested that the inhibition of these arousal pathways by neurons in the lateral hypothalamus and the hypothalamic ventrolateral preoptic nucleus (VLPO) ultimately promotes sleep. The sleep-promoting nucleus VLPO appears to play a prominent role in both NREM and REM sleep. Lesions of the VLPO cluster significantly reduced NREM sleep, while lesioning of the extended VLPO resulted in a reduction in REM sleep. The inhibitory relationship between the VLPO and the ascending reticular formation was found to be reciprocal.

3.2. Stress-induced alterations in sleep in adulthood

It has long been known that stress can modulate sleep and can be an important cause of sleep disturbance. Indeed, most studies in rodents show an initial suppression of NREM sleep and/or REM sleep in response to acute stress exposure, subsequently followed by an increase in sleep time or sleep rebound (Meerlo et al., 1997; Meerlo et al., 2001; Koehl et al., 2002; Tiba et al., 2003; Koehl et al., 2006; Tang et al., 2007). However, the presence of such stress-induced alterations in sleep patterns appears to depend on the nature and

controllability of the stressor (Papale et al., 2005; Sanford et al., 2010) as well as the strain of mice or rats used (Meerlo et al., 2001; Sanford et al., 2003; Tang et al., 2005).

Some of these stress-induced alterations in sleep-wake behavior may be mediated by stress hormones. The nocturnal rhythm of the human glucocorticoid cortisol appeared to be related to sleep architecture. During light sleep and wakefulness increasing cortisol levels were observed, while REM sleep was associated with decreasing cortisol concentrations (Born et al., 1986). The acute administration of a synthetic glucocorticoid significantly reduced REM sleep in healthy subjects (Gillin et al., 1972; Born et al., 1987). In line with this, the administration of ACTH provoked an increase in light sleep and wakefulness, suggested to be mediated indirectly by an increase of cortisol (Follenius et al., 1985). However, Steiger and colleagues reported that a synthetic ACTH analog, lacking effects on cortisol, reduced the overall time spent asleep, suggesting direct effects of ACTH on sleep (Steiger et al., 1991). In addition, CRH administration decreased SWS and REM sleep, while increasing light sleep in healthy male subjects (Holsboer et al., 1988). Similarly, in male rats acute corticosterone administration increased wakefulness at the expense of time spent in NREM sleep, while REM sleep duration remained unaffected (Vázquez-Palacios et al., 2001). Furthermore, the acute administration of CRH increased the time spent awake and reduced both REM and NREM sleep duration in male rodents, while the administration of a CRH receptor antagonist reduced wakefulness and increased NREM sleep duration in male rats (Chang & Opp, 1998; Romanowski et al., 2010).

3.3. Early life influences on sleep

During the first postnatal month sleep architecture differs substantially from the sleep-wake patterns observed during adulthood (Jouvet-Mounier et al., 1969; Frank & Heller, 1997a; Frank & Heller, 1997b). In general, infant rats spend more time asleep than adult animals. During the early postnatal period sleep only consists of REM sleep, SWS firstly appears after approximately postnatal day 10, simultaneously with a decrease in the amount of REM sleep per day. At the age of 1 month the sleep-wake behavior of the young rats is similar to the patterns reported in adulthood. Adult rats spend the majority of the time either awake or in NREM sleep, only about ten percent of the day is devoted to REM sleep.

During early life, sleep was found to be under maternal regulation. Separating mother and pups at two weeks of age increased pup activity and wakefulness and altered sleep architecture, or more specifically maternal separation caused sleep fragmentation, delayed REM sleep onset and decreased time spent in REM sleep (Hofer, 1973; Hofer, 1976). Maternal separation was also found to have long-lasting effects on sleep-wake behavior. Rats that were deprived of their mother for 3 hours per day during the first two postnatal weeks had different sleep-patterns in adulthood, particularly they exhibited a higher sleep efficiency and increased REM sleep duration and bouts during daytime when compared to controls (Tiba et al., 2004). In contrast, the brief maternal absence during early handling did not alter adult sleep patterns (Tiba et al., 2003).

The previously mentioned acute changes in sleep-wake patterns as a consequence of early life adversity may affect brain development. Roffwarg and colleagues were the first

to suggest that sleep, and more specifically REM sleep, might be essential for brain maturation (Roffwarg et al., 1966). Although, it is suggested that both REM and NREM sleep influence brain development, most studies focused on the role of REM sleep. REM sleep deprivation, induced by a platform-over-water method, was found to delay normal brain development (Shaffery et al., 2002). Pharmacological REM sleep deprivation during early life was found to alter e.g. exploratory and sexual behavior during adulthood and reduce the size of the cerebral cortex and medulla oblongata (Mirmiran et al., 1981; Mirmiran et al., 1983). However, Mirmiran and colleagues suggested that these alterations might also be caused by changes in certain neurotransmitter systems as an immediate result of the pharmacological treatment.

3.4. Sex differences in sleep-wake behavior

Despite similar baseline sleep patterns in men and women, epidemiological studies reported clear sex differences in the development of sleep disorders and the prevalence of sleep complaints (e.g. Krishnan & Collop, 2006; Dijk et al., 1989; Goel et al., 2005). Similar to humans, the male-female differences in baseline sleep patterns in rodents are often rather subtle. Some studies showed significantly more REM sleep in males compared to females (Yamaoka, 1980; Fang & Fishbein, 1996; Tiba et al., 2008), while others showed higher slow wave activity (SWA), an indicator of sleep intensity, in females (Ehlers et al., 1993) or significantly higher levels of nocturnal NREM sleep specifically in diestrus females (Andersen et al., 2008) when compared to male rats. Similarly, male mice exhibit slightly more NREM sleep (Koehl et al., 2006; Paul et al., 2006) and REM sleep (Koehl et al., 2006) when compared to females. The sex differences in sleep architecture observed in these papers are likely related to the influence of sex steroids, since gonadectomy in male and female mice was found to eliminate the differences in sleep-wake behavior (Paul et al., 2006). Several studies described estrous cycle-related differences in sleep patterns. While several papers reported a reduction in REM sleep during the proestrus phase in female rats (Zhang et al., 1995; Fang & Fishbein, 1996; Schwierin et al., 1998; Hadjimarkou et al., 2008), part of which show a concomitant reduction in NREM sleep (Zhang et al., 1995; Schwierin et al., 1998; Hadjimarkou et al., 2008), another rat study exhibited no cycle-related differences (Andersen et al., 2008). In a study by Koehl and colleagues, estrous phase differences in mice were shown to be strain dependent, with either subtle differences in NREM and REM sleep or similar sleep patterns when comparing different stages (Koehl et al., 2003). Furthermore, ovariectomy was found to increase nocturnal REM sleep, whereas REM sleep was reduced by estradiol administration in ovariectomized female rats (Colvin et al., 1969; Fang & Fishbein, 1996; Deurveilher et al., 2009). Similarly, progesterone reduced REM sleep (Lancel et al., 1996; Deurveilher et al., 2009) and furthermore dose-dependently increased REM latency and decreased NREM latency (Lancel et al., 1996). In contrast, another study reported a decreased REM latency in both male and female rats after progesterone injection directly into the reticular formation (Camacho-Arroyo et al., 1999).

Studies on the effects of testosterone on sleep architecture are rather scarce. Gonadectomy did either increase nocturnal REM sleep in rats (Yamaoka, 1980) or had no

effect on sleep-wake behavior (Peder, 1987). Mice implanted with continuous testosterone releasing pellets spent significantly more time in NREM sleep during the dark phase when compared to placebo treated mice (Paul et al., 2009).

4. Hippocampal neurogenesis

4.1 Adult neurogenesis

Differences in adult stress systems, which might be caused by amongst others developmental programming and sex hormones, may ultimately affect the susceptibility to stress-related disorders such as depression. This difference in sensitivity for psychopathology might be related to stress-induced alterations in brain plasticity and neurogenesis.

It has been long assumed that neurogenesis, or the production of new neurons, only occurs during development (Ramon y Cajal, 1928; Gross, 2000). However, in the 1960s, the presence of newborn neurons in the adult rat brain was first demonstrated (Altman, 1962). The two main areas in the brain exhibiting ongoing neurogenesis in adulthood are the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ), from which cells migrate to the olfactory bulb (Altman & Das, 1965; Altman, 1969; Kaplan & Hinds, 1977). Considering the focus of the present thesis, a more detailed description of specifically hippocampal neurogenesis will follow below.

The hippocampus, part of the limbic system, plays, as previously mentioned, a prominent role in the negative feedback regulation of the HPA-axis, but is also involved in cognitive function and emotional regulation (Jarrard, 1993; Moser and Moser, 1998). In the hippocampus different subregions can be distinguished, that is, the dentate gyrus (DG) and the cornu ammonis (CA) regions 1, 2 and 3. The major innervation of the hippocampus comes from the entorhinal cortex. Besides direct projections from the entorhinal cortex to the different subregions of the hippocampal formation, input is transferred to the other subregions by a unidirectional route that connects the different subregions (figure 2; for review, see van Strien et al., 2009).

The DG consists of different cell layers, a granule cell layer, a molecular layer - in which the dendrites of the granule cells are located - and a polymorphic layer or hilus, which is enclosed by the granule cell layer and contains the axons of the granule cells that project to the CA3 area (figure 2; for review on dentate gyrus organization, see Amaral et al., 2007). The bundle of axons projecting from the granule cells to the CA3 area is also referred to as the mossy fiber pathway. Proliferating stem cells that give rise to new neurons are located in the subgranular zone, situated between the granule cell layer and the hilus. It is suggested that per day about 9,000 new cells are generated in the DG of young adult rats (Cameron & McKay, 2001), a number equal to about 1% of the total granule cell population (Boss et al., 1985). These new born cells migrate into the granule cell layer, where some of them ultimately mature and become functionally incorporated. Between 4 until 10 days after formation, new born neurons start to extend axons towards the hippocampal CA3 area (Stanfield & Trice, 1988; Hastings & Gould, 1999). After about 4 weeks, the newly formed neurons are functionally incorporated, showing

electrophysiological and morphological properties of mature granule cells (van Praag et al., 2002).

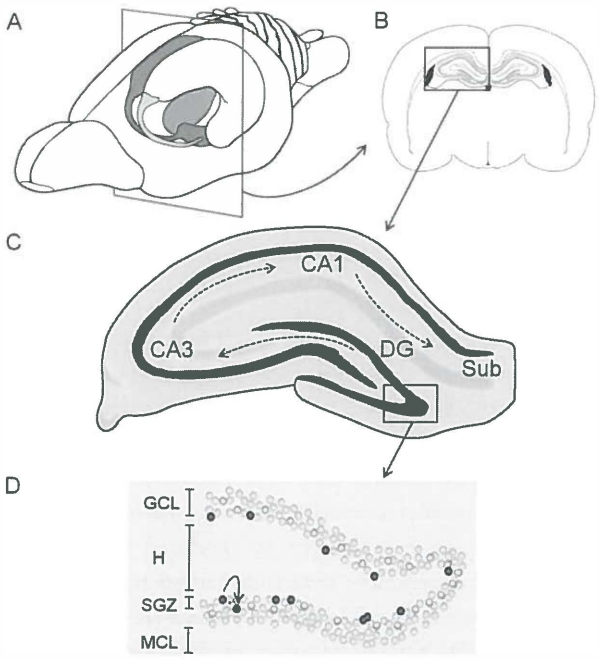


Figure 2 *Position and structure of the hippocampus. A) location of the hippocampus in the rat brain; B) coronal section showing the dorsal hippocampus; C) Illustration of the unidirectional route connecting the different subregions of the hippocampus (DG: dentate gyrus; Sub: subiculum); D) Organization of the dentate gyrus (MCL: molecular cell layer, GCL: granule cell layer, H: hilus, SGZ: subgranular zone). The newly born cells in the SGZ migrate into the GCL (arrow), where the young neurons are functionally incorporated.*

The rate of hippocampal neurogenesis is not stable, but can be modulated by a wide variety of factors (Lucassen et al., 2010). Exercise and environmental enrichment were found to be positive regulators and enhance hippocampal neurogenesis (Nilsson et al., 1999; van Praag et al., 1999). In contrast, ageing, sleep disturbances and stress suppress dentate neurogenesis (Kuhn et al., 1996; Gould et al., 1997; Meerlo et al., 2009). The influence of stress on neurogenesis will be described in more detail in the next paragraph.

Although the exact function of the newborn granule neurons remains to be determined, it is suggested that neurogenesis is involved in cognition and emotional functioning (Korosi et al., 2011). While exercise-induced increases in neurogenesis improved learning performance, a decline in neurogenesis resulting from e.g. treatment with an antimitotic agent (methylazoxymethanol acetate or MAM) reduced performance in certain hippocampus-dependent tasks (van Praag et al., 1999; Shors et al., 2002).

Furthermore, the dysregulation of neurogenesis has been implicated in the development of mood disorders such as depression (Lucassen et al., 2010).

4.2. Stress-induced alterations in adult neuroplasticity

Both acute and repeated or chronic stressful experiences demonstrated an inhibitory influence on different phases of hippocampal neurogenesis (Gould et al., 1997; Tanapat et al., 2001; Pham et al., 2003). However, another study suggested that changes in neurogenesis may only occur after chronic stress exposure (Dagyte et al., 2009). Furthermore, the acute administration of corticosterone reduced the number of new born cells, whereas the removal of endogenous adrenal steroids resulted in increased cell proliferation levels (Cameron & Gould, 1994). Glucocorticoids might also play a role in the ageing-related decrease in neurogenesis, since adrenalectomy was found to prevent the age-associated decline (Cameron & McKay, 1999).

The influence of adrenal steroids on the production of new granule cells might be mediated directly by GR and MR. Cameron and colleagues found that some hippocampal progenitor cells show clear GR expression 1h after division and MR expression after 24 hours (Cameron et al., 1993). In contrast, another study suggests that MR expression is only first seen 3 days after division, while GRs were already observed after 4h (Garcia et al., 2004). It was suggested that, besides a possible direct regulatory role of corticosterone, the stress-induced effects on neurogenesis might be mediated by NMDA-receptor activity (Gould et al., 1997). Stressful experiences were found to up-regulate hippocampal NMDA-receptor mRNA, although only in the CA1 and CA3 area (Krugers et al., 1993; Bartanusz et al., 1995). Activation of the NMDA-receptor inhibits hippocampal cell proliferation, whereas reduced NMDA-receptor activation by pharmacological blockage increases the number of new born cells (Cameron et al., 1995).

Chronic glucocorticoid exposure may not only reduce neurogenesis in the DG but may also lead to cell death in other hippocampal regions, particularly in the CA3 area (Sapolsky et al., 1985). Furthermore, chronic glucocorticoid or repeated stress exposure was found to alter the apical dendritic morphology of the CA3 pyramidal cells (Woolley et al., 1990a; Watanabe et al., 1992; Galea et al., 1997). However, these alterations appear to be only temporary, given that about one week after stress termination the apical dendrites returned to baseline levels (Galea et al., 1997). A more recent study reported that the severity of the stress-induced alterations in the CA3 pyramidal cell dendrites might be dependent on type of stressor. Whereas chronic restraint stress induced clear atrophy of both apical and basal dendrites, chronic unpredictable stress, consisting of 8 different stressors, had a less pronounced effect on, mainly apical, dendritic morphology (Vyas et al., 2002).

4.3. Early life influences on neuroplasticity

The development of the rodent hippocampus is still ongoing well into early postnatal life (Angevine, 1965). The dentate gyrus develops in different migratory phases, during which cells move from the subventricular zone towards the dentate gyrus. During this process a neurogenic zone in the hilus, from where neural progenitor cells originate to build the

subgranular zone, and the granular cell layer are formed. A clear peak in subgranular zone neurogenesis was observed at the beginning of the third postnatal week in rats (Altman & Das, 1965). During the first postnatal weeks also synaptogenesis is increased and connectivity patterns are established. For example, mossy cell dendrites start to show a mature appearance by postnatal day 21 (Ribak et al., 1985). Furthermore, at postnatal day 4 less than 1 percent of the adult synapse number is present in the molecular layer of the DG, which doubles every day between postnatal day 4-11 (Crain et al., 1973). Eventually, at postnatal day 30 the development of the dentate gyrus is completed (for a more detailed overview, see Altman & Bayer, 1990a & 1990b).

Early life experiences might alter or disrupt the structural and functional maturation of the hippocampal network. Indeed, early life events were found to have a long-lasting effect on hippocampal plasticity and function. Dependent on the severity of the stress exposed to in early life, adult hippocampal function might be either enhanced or impaired. While handling, a procedure considered to be a mild early life stressor, improved hippocampal-dependent learning and memory in adulthood (Meaney et al., 1988; Fenoglio et al., 2005), some studies suggest that maternal separation, a more severe early life stress, impairs learning capacity in the adult offspring (Oitzl et al., 2000; Huot et al., 2002; Aisa et al., 2007). The latter, however, was not confirmed in all studies (Grace et al., 2009).

In the few papers focusing on handling-induced hippocampal alterations, handling did not substantially alter adult neurogenesis (Lemaire et al., 2006) or dendritic morphology in the hippocampal CA1 area (Monroy et al., 2010). In contrast, different structural alterations of the hippocampus were observed after maternal separation. Adult maternally-separated rats showed either a decrease in cell proliferation (Aisa et al., 2009; Mirescu et al., 2004) or similar cell proliferation and survival levels as controls (Mirescu et al., 2004; Nair et al., 2007). Furthermore, MS was suggested to decrease dendritic length of CA1 pyramidal cells (Monroy et al., 2010), reduce mossy fiber density (Huot et al., 2002) and attenuate synapse formation (Andersen & Teicher, 2004).

4.4. Sex differences in neuroplasticity

Several studies reported on adult sex differences in hippocampal structure and function. Male-female differences have been observed in long-term potentiation and hippocampal-dependent learning, with better performance in male rats than females (Roof & Havens, 1992; Maren et al., 1994; Isgor & Sengelaub, 1998). The performance in a spatial learning task was suggested to be positively correlated with the size of the dentate granule cell layer (Roof & Havens, 1992). While some studies reported that males have a significantly larger granule cell layer (Roof & Havens, 1992; Roof, 1993) and a greater number of granule neurons when compared to females (Madeira et al., 1988), other studies suggested that there is no sex difference in dentate granule cell layer volume (Isgor & Sengelaub, 1998; Jones & Watson, 2005). The volume of the CA1 and CA3 area was found to be approximately 20% smaller in female rats when compared to males (Isgor & Sengelaub, 1998). With respect to sex differences in neurogenesis, higher levels of cell proliferation have been reported in females by Tanapat and colleagues (1999), but not by others (Falconer & Galea, 2003; Shors et al., 2007; Brummelte & Galea, 2010). No male-female

differences were observed in the differentiation or survival of adult-born cells (Tanapat et al., 1999; Brummelte & Galea, 2010). On the other hand, females show a greater spine density in the CA1 area and a higher number of synapses in the CA3 area of the hippocampus, whereas males show a higher mossy fiber synapse density in the hilar region of the dentate gyrus (Madeira & Paula-Barbosa, 1993; Parducz & Garcia-Segura, 1993; Shors et al., 2001).

Some of the male-female differences in hippocampal structural plasticity might be mediated by or dependent on the presence of circulating gonadal hormones. Indeed, the naturally occurring fluctuations in estrogen and progesterone across the estrous cycle in female rodents dramatically influence neurogenesis and dendritic morphology of CA1 pyramidal cells. While the spine density of the CA3 pyramidal cells and the dentate gyrus granule neurons is stable across the cycle, the spine and synapse density in the CA1 area is clearly correlated with the changing estradiol and progesterone levels. The highest dendritic spine and synapse density is present during the proestrus phase of the estrous cycle, when estradiol and progesterone levels peak, whilst during the estrus phase the density was about 30% lower (Nequin et al., 1979; Woolley et al., 1990b; Woolley & McEwen, 1992). Furthermore, short-term ovariectomy decreased dendritic spine density of the pyramidal cells in the CA1 area, while treatment with estradiol was found to reverse the OVX-induced decrease in CA1 spine density, an effect which was enhanced by the administration of progesterone (Gould et al., 1990). However, progesterone shows a clear biphasic effect by initially increasing and subsequently sharply decreasing spine density in estradiol treated rats. The dramatic and rapid proestrus to estrus decline in spine density was found to be mainly attributable to progesterone (Woolley & McEwen, 1993).

Androgens were also found to influence CA1 pyramidal cell spine synapse density. In male rats, gonadectomy (GNX) strongly reduces synapse density in the CA1 region by approx. 50%, whereas treatment with testosterone reversed this GNX-induced decrease in spine synapse density (Leranth et al., 2003).

Neurogenesis has also been shown to be under the control of sex hormones. The rate of hippocampal cell proliferation in female rats is dependent on the stage of the estrous cycle, a relationship associated with the levels of estradiol and progesterone. Female rats produce significantly more new cells during proestrus compared to diestrus or estrus (Tanapat et al., 1999). Furthermore, ovariectomy reduced hippocampal cell proliferation, which was completely reversed by estrogen replacement (Tanapat et al., 1999; Banasr et al., 2001). While estradiol initially enhances cell proliferation, a more prolonged exposure for 48h suppressed the production of new born cells in female rats. It was suggested that this estradiol-induced suppression is mediated, at least in part, by adrenal steroids, since adrenalectomy prevented the estradiol-induced decrease in cell proliferation (Ormerod et al., 2003). Furthermore, repeated administration of estradiol for 15 days decreased cell survival in OVX female rats (Barker & Galea, 2008). Progesterone, which is naturally present in both males and females, has also been shown to influence hippocampal cell proliferation. Subsequent administration of estradiol and progesterone in OVX-female rats inhibited the estradiol-induced increase in cell proliferation (Tanapat et al., 2005). The administration of exclusively progesterone enhanced the survival of new born cells in male

mice (Zhang et al., 2010). However, a more recent study in male rats showed a clear progesterone-induced increase in cell proliferation, but not cell survival (Barha et al., 2011). The influence of androgens on hippocampal neurogenesis is considerably less studied. Gonadectomy did not affect cell proliferation, but significantly reduced cell survival in the DG of adult males, whereas testosterone up-regulated cell survival in gonadectomized male rats (Spritzer & Galea, 2007). Another study reported a decrease in cell survival after the administration of the testosterone analogue 19-nortestosterone in intact rats (Brännvall et al., 2005).

5. Outline of the thesis

Recapitulating, adverse early life experiences and sex might alter adult stress systems, sleep and neuroplasticity, which in turn may influence the susceptibility to the development of psychopathologies like depression. The aim of the studies described in this thesis was to investigate the influence of early stressful life events and sex on adult stress sensitivity. This thesis consists of two main parts. The first part focuses on the consequences of early life stress, or more specific maternal separation, on adult neuroendocrine and cardiovascular stress reactivity, as well as adult stress-induced changes in neurogenesis. The second part describes studies on male-female differences in stress sensitivity, with emphasis on stress-induced alterations in sleep and neurogenesis.

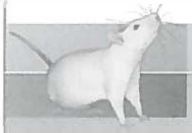
In chapters 2 and 3, the long term effects of maternal separation on adult stress responsivity, neuroplasticity and behavior were studied. For this purpose, mother and pups were separated for 3 hours during the first two postnatal weeks, coinciding with the sensitive postnatal developmental period. During adulthood, stress-induced alterations in HPA-axis regulation and cardiovascular system function and structure were investigated. Furthermore, the influence of maternal separation per se, and in combination with adult stress, on neurogenesis were studied. Considering that alterations in stress systems and neurogenesis may affect normal brain function and have been implicated in the development of mood disorders, changes in cognitive functioning and anxiety-like behavior were assessed.

Whereas only minor male-female differences in basal sleep-wake patterns are reported, stress might enhance these initially subtle differences. The male-female differences in stress-sensitivity and reactivity might also be related to the sexually biased prevalence of sleep disorders and complaints. Therefore, the main aim in chapter 4 was to investigate whether the exposure to an emotional or physical stressor induced sexually dimorphic alterations in sleep architecture.

While available data suggests subtle sex differences in hippocampal plasticity and neurogenesis, it is uncertain whether sex also modulates the effects of stress on neurogenesis. In the last experimental chapter, chapter 5, the male-female differences in stress-induced alterations in neurogenesis were studied. Furthermore, the sex differences in neuroendocrine stress reactivity were assessed in response to a variety of stressors.

Finally, in the last chapter, the general discussion, the results from the different chapters will be summarized and discussed.

2.



Early maternal separation has mild effects on cardiac autonomic balance and heart structure in adult male rats

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Abstract

Early life adverse experiences have long-term physiological and behavioral effects and enhance stress sensitivity. This study examined the effects of maternal separation (MS) on cardiac stress responsivity and structure in adulthood. Male Wistar rats were separated from the dams for 3 h per day from postnatal day 2 through 15. When exposed to 5-day intermittent restraint stress (IRS) as adults, MS and control rats showed similar acute modifications of cardiac sympathovagal balance, quantified via heart rate variability analysis. In addition, MS had no effect on cardiac pacemaker intrinsic activity (as revealed by autonomic blockade with scopolamine and atenolol) and did not affect the circadian rhythmicity of heart rate, neither before nor after IRS. However, MS differed from control rats in cardiac parasympathetic drive following IRS, which was heightened in the latter but remained unchanged in the former, both during the light and dark phases of the daily rhythm. The evaluation of adult cardiac structure indicated that stress experienced during a crucial developmental period induced only modest changes, involving cardiomyocite hypertrophy, increased density of vascular structures, and myocardial fibrosis. The mildness of these functional-structural effects questions the validity of MS as a model for early stress-induced cardiac disease in humans.

1. Introduction

It is widely accepted that stressful events occurring early during postnatal life may alter neuroendocrine and behavioral stress responsiveness and lead to greater susceptibility for psychopathology throughout life (Heim and Nemeroff, 2001; Cirulli et al., 2009).

Animal models based on the disruption of mother-infant relationship have been used for a long time to better understand the short- and long-term effects of early adverse experiences (Lehmann and Feldon, 2000; Levine, 2001; Faturi et al., 2010). In rodents, one of the most commonly used experimental paradigms of early life stress is maternal separation (MS), in which pups are removed from maternal nest repeatedly for a variable time period during the lactation phase. Brief maternal separation (3-15 min per day for several days), also termed early handling (EH), has rather robust developmental effects and leads to attenuated adrenal and behavioral responses to stress in adulthood (Levine, 1957; Levine et al., 1967; Meerlo et al., 1999). However, prolonged MS (3h or more per day, for several days) has long-term effects that appear to be highly variable, depending on details of the procedures and rat strains used (for review, see Lehmann and Feldon, 2000). A number of studies indicate that animals subjected to MS develop a phenotype that is opposite to that of individuals exposed to EH (Plotsky and Meaney, 1993; Meaney et al., 1996). In particular, maternally separated rats were reported to display greater hypothalamic-pituitary-adrenal (HPA) axis reactivity to acute challenges and higher levels of anxiety in adulthood (Huot et al., 2002; Plotsky et al., 2005; Aisa et al., 2007), potentially resulting in a larger vulnerability to stress-related disease. However, other studies have reported no major effects of MS on adult adrenocortical activity and anxiety-like behavior (Slotten et al., 2006; Hulshof et al., 2011).

In recent years, there has been a growing interest in the effects of early adverse experiences on cardiovascular system function and structure. Individual features of cardiovascular regulation result from a dynamic interaction between genetically programmed developmental processes and environmental conditions (Tucker et al., 1984). During ontogeny, cardiac development and maturation partly depend on, and interact with, input from the autonomic nervous system (ANS; Claycomb, 1976; Larson and Porges, 1982; Tucker and Johnson, 1984a; Tucker, 1985). For these reasons, stress experienced in this crucial phase may interfere with normal autonomic fiber distribution to the myocardial tissue, which in turn might lead to persistent changes in the functional and morphological characteristics of the heart (Tucker et al., 1984; Tucker and Johnson, 1984b).

In humans, epidemiological evidence indicates that unfavourable events experienced early in life are associated with an increased susceptibility to develop heart disease in adulthood. For instance, the Adverse Childhood Experiences Study reported that childhood abuse and neglect are closely associated with the most important risk factors, such as smoking, obesity, physical inactivity and depression, for ischemic heart disease, (Dong et al., 2004). However, such epidemiological studies provide no evidence for causal relationships and so far only few experimental studies with animal models have investigated the direct link between early adverse experiences and cardiovascular (dys-)function (Sanders and Anticevic, 2007; Loria et al., 2010a,b). On the one hand, results of these

studies suggest that MS does not influence baseline values of heart rate and blood pressure. On the other hand, in one study, MS increased heart rate responsivity to an acute stressor in adult borderline hypertensive rats, with no significant changes in blood pressure response (Sanders and Anticevic, 2007). Furthermore, early life stress rendered adult rats more susceptible to angiotensin II-induced hypertension, tachycardia and vascular inflammation, which may contribute to the pathogenesis of cardiovascular disease (Loria et al., 2010a,b). Altogether, the data collected so far indicate that MS may contribute to adult cardiovascular morbidity; nonetheless, it is still unknown whether the autonomic control of cardiac function is affected, at rest and during exposure to acute environmental stimuli and whether the early life adverse experience of MS may alter the development of adult heart morphology and tissue anatomy.

Studies in both humans and animals report that impaired cardiac autonomic regulation characterizes many pathological conditions of cardiac (Brook and Julius, 2000; Thayer et al., 2010) and non-cardiac origin (Ewing et al., 1985; Thayer et al., 1996). In humans, the analysis of heart rate variability (HRV), which describes the small beat-to-beat differences in heart rate is a non-invasive approach to gather information about the modulation of the two branches of the ANS to the heart (Task Force, 1996). The same approach has also been successfully applied to rat electrocardiogram (ECG) recordings (Sgoifo et al., 1998; Aubert et al., 1999).

In this study, we tested the hypothesis that adverse events experienced early during postnatal life may interfere with the development of the heart and its autonomic neural control, possibly leading to an increased stress susceptibility in adulthood. In particular, we assessed whether MS in rats induces long-lasting modifications of: (i) cardiac autonomic regulation, both at rest and under challenging conditions, namely restraint stress and pharmacological autonomic blockade, and (ii) cardiac architecture, in terms of gross morphological changes, vascular density, and myocardial structural damage.

2. Materials & methods

2.1 Animals and housing

Twenty-four male Wistar rats were used in this study. All efforts were made to reduce animal pain or discomfort. Experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the University of Parma Animal Welfare Committee. Female and male Wistar rats (Charles River, Calco, Italy) were paired for a period of 10 days, after which the males were removed. Subsequently, pregnant females were left undisturbed, except for a daily visual check for the presence of pups. The day of birth was defined as pups being present by 10:00h and was designated as day 0. On postnatal day 1, the litters were culled to eight pups, four males and four females. At weaning (3 weeks of age), they were housed with same-sex siblings. No more than two male pups from each litter were used for any given measure. During the entire experiment, all rats were kept under controlled temperature (22

± 2 °C) and lighting (lights on from 07:00h to 19:00h) conditions. Ad libitum access to food and water was provided throughout the study.

2.2. Maternal separation

MS, which consisted of daily separation of the litter from the dam for 3 h (9:00h-12:00h), was carried out from postnatal day 2 to postnatal day 15, in accordance with previous studies (Plotsky and Meaney, 1993, Hulshof et al., 2011). Each litter was removed from the nest and transferred to another room, to prevent vocal communication between mother and pups. During this 3h period, the pups were placed with their siblings in glass beakers in a water bath set at 32-33°C, consistent with normal nest temperature (Schmidt et al., 1986), in order to prevent a decrease in body temperature. A reduction in body temperature of the litter has been shown to increase maternal care upon reunion (Leon et al., 1978; Stern and Johnson, 1990), which was found to reduce behavioral and neuroendocrine stress reactivity (Liu et al., 1997; Francis et al., 1999).

During the separation period, the dams remained in the home-cage. Following the 3h MS period, pups were returned to the home cage. Control pups belonged to other (independent) litters and were left undisturbed throughout the pre-weaning period in their mother's nest.

2.3. Radiotelemetry system

The radiotelemetry system employed in this study consisted of flat transmitters measuring 25 x 15 x 8 mm (TA11CTA–F40, Data Science International, St. Paul, MN, USA) and platform receivers. At 4 months of age, the animals were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil, 200 mg/kg, s.c.) and the transmitters chronically implanted according to a surgical procedure that guarantees high-quality ECG recordings also during sustained physical activity (Sgoifo et al., 1996). Immediately after surgery, rats were individually housed and injected for 2 days with gentamicine sulfate (Aagent, Fatro, 0.2 ml/kg, s.c.). The rats were allowed 10 days of recovery before the start of experimental recordings.

2.4. Outline of adult manipulations

An overview and timeline of the measurements that were carried out in adulthood is provided in Figure 1. Four-month-old MS (n=12) and control (n=12) rats were exposed to an intermittent restraint stress (IRS) protocol. Rats were subjected to restraint stress on 5 consecutive days. Each restraint session consisted of confinement in a wire-mesh tube for 15 min (inner diameter 6 cm, length 20 cm). Eight days before and after IRS protocol, pharmacological blockade of the two branches of the ANS was carried out (see below for details). The weeks in between the pharmacological tests and the IRS protocol were used to assess the daily rhythmicity of heart rate. From the day of surgery, the animals were weighed on a weekly basis until the day of euthanasia.

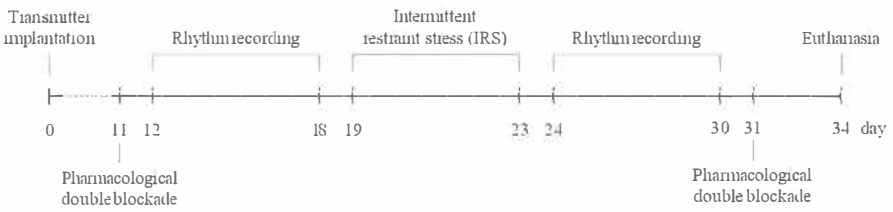

Figure 1 *Schematic diagram of the experimental protocol applied to control and maternally separated rats in adulthood. Transmitter implantation, the surgical chronic implantation of transmitters for radiotelemetric ECG recordings. Pharmacological double blockade, pharmacological blockade of the two branches of the ANS with methylscopolamine (muscarinic receptor antagonist) and atenolol (beta-adrenergic receptor antagonist). IRS, daily exposure to restraint stress for 5 consecutive days. Rhythm recording, around-the-clock, radiotelemetric ECG recording for heart rate rhythmicity evaluation. Euthanasia, euthanasia of the rats and subsequent removal of hearts and adrenals.*

2.5. Pharmacological autonomic blockade

The competitive muscarinic receptor antagonist methylscopolamine (0.05 mg/kg) and sympathetic blocker atenolol (2 mg/kg; Sigma, St.Louis, MO, USA; Ngampramuan et al., 2008) were injected s.c. to block vagal and sympathetic influences to the heart in MS and control animals. After baseline ECG recording, methylscopolamine was injected and the ECG recorded to evaluate the effect of parasympathetic blockade; 15 min afterwards, atenolol was administered to the same rats to determine intrinsic heart rate. Intrinsic heart rate is established when the cardiac ANS is completely blocked, which is supposed to take place approximately 10-15 min after the sympathetic blocker injection (Safa-Tisseront et al., 1998; Souza et al., 2009; Sant'Ana et al., 2011). The pharmacological test was carried out twice, 8 days before and 8 days after IRS (Figure 1).

2.6. Electrocardiographic data collection and analysis

ECG waves were acquired on a personal computer via the ART-Silver 1.10 data acquisition system (Data Sciences Int., St. Paul, MN, USA) with 1000 Hz sampling frequency. Continuous ECG recordings were carried out during the first and fifth restraint stress episodes and the two pharmacological tests (Figure 1) according to the following schedule: (i) restraint stress: 30 min baseline, 15 min test, 30 min recovery and (ii) pharmacological double blockade: 30 min baseline, 15 min following scopolamine injection, 45 min following atenolol injection. Chart5 software (ADInstruments, Sydney, Australia) was employed to calculate the average R-R interval duration (RR, ms), which corresponds to the average inter-beat interval in a given time period. In addition, time- and frequency-domain parameters of HRV were quantified. The time-domain indexes used in this study were the root mean square of successive differences between adjacent R-R intervals (r-MSSD, ms) and the percentage of successive interval differences larger than 20 ms

(pNN20, %). R-MSSD and pNN20 quantify short-term, high-frequency (HF) variations of RR and therefore estimate the activity of the parasympathetic nervous system (Stein et al.,1994). Frequency-domain (fast Fourier transform) indices were collected in accordance with the guidelines for frequency-domain computations of HRV (Task Force, 1996). We considered only low frequency (LF; 0.2-0.75 Hz) and HF (0.75-2.5 Hz) bands of the spectrum, and their power was quantified as normalized units (n.u.). The power of LF represents the activity of both branches of the ANS (Eckeberg, 1991); the power of HF is due to the activity of the parasympathetic nervous system and includes respiration-linked oscillations of heart rate (Chess et al., 1975). The LF/HF ratio estimates the fractional distribution of power, which is taken as an indirect measure of sympathovagal balance (Task Force, 1996). A stationary ECG signal is recommended to reliably carry out short-term frequency-domain HRV analysis and the presence of artifacts could significantly influence the results (Task Force, 1996). For these reasons, those parts of ECG recordings that were non-stationary and/or exhibited recording artifacts were excluded from the analysis.

2.7. Daily rhythm data collection and analysis

ECG waves were sampled around-the-clock for 60 s every 60 min in two different periods: (i) pre-IRS, for 7 days, starting on the day after the first pharmacological test, and (ii) post-IRS, for 7 days, starting on the day after the last restraint test (Figure 1). ECG recordings were analyzed by means of a software package developed in our laboratory (Sgoifo et al., 2001) and RR and r-MSSD values were quantified as means for the 12-h light (resting) phase and 12-h dark (activity) phase.

2.8. Post-mortem measurements

Three days after post-IRS pharmacological blockade (Figure 1), rats were euthanised. Under anesthesia (tiletamine hydrochloride + zolazepam hydrochloride, Zoletil, 200 mg/kg, s.c.) the heart was arrested in diastole with cadmium chloride solution (100 mM IV) and excised for subsequent morphological/morphometric analysis. Following heart removal, the adrenals were also excised and weighed. Adrenal glands were also obtained from 20 additional 5-month-old rats (MS, n=10; control, n=10), which were not exposed to adult experimental manipulation.

Cardiac remodelling The two atria, the right ventricle (RV) and the left ventricle (LV) inclusive of the septum were separately weighed, fixed in 10% buffered formalin solution, and used for morphometric studies. The following parameters were determined: heart weight (HW), LV weight (LVW), RV weight (RVW), LVW/HW and RVW/HW. LV free wall thickness and LV transverse diameters were morphometrically computed (Image Pro-plus). The LV chamber volume was calculated according to the Dodge equation (Dodge and Baxley, 1969). From each heart embedded in paraffin, 5 μm-thick left ventricular sections were cut from the equatorial slice and used for subsequent analyses.

Morphometric analysis Sections stained with Masson's trichrome were analyzed by optical microscopy (magnification 250X) in order to evaluate the total amount of interstitial and reparative fibrosis (Beltrami et al., 1994) in the LV myocardium. According to a procedure previously described (Costoli et al., 2004), quantitative evaluation of fibrotic tissue was carried out in 60 randomly selected fields from the subendocardium, midmyocardium and subepicardium, with the aid of a grid defining a tissue area of 0.160 mm^2 and containing 42 sampling points, each covering an area of 0.0038 mm^2. To define the volume fraction of fibrosis in the three layers of the ventricular wall, the number of points overlaying myocardial scarring was counted and expressed as a percentage of the total number of points explored. For reparative fibrosis, the numerical density of fibrotic foci per unit area of myocardium was also determined.

Myocyte size The cross-sectional area (CSA) of myocytes was determined by measuring the cell diameter in transversally oriented myocytes, but only in those cells where the entire nuclear profile was clearly defined. To obtain CSA, two diameters were measured and their mean value was used to compute the area. For each LV, 120-250 cardiomyocytes were analyzed at a magnification of X1000.

Vessel density The quantification of capillaries and venules was carried out in sections stained with polyclonal rabbit anti-vW factor (dilution 1:100, Dako), which recognizes endothelial cells, followed by fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-rabbit secondary antibodies (Jackson Laboratory, Baltimore, PA, USA). Nuclei were recognized by bisbenzimide staining (Hoechst No. 33258, Sigma, St Louis, MO, USA). Morphometric sampling at X1000 magnification consisted of counting the number of capillary and venule profiles in a measured area of tissue sections of both the epimyocardium and endomyocardium, in which myocytes are transversally oriented. Capillaries were distinguished according to their luminal diameter (range 4-6 μm) from venules (range 6-10 μm) in which vW factor positive profiles lack multiple layers of smooth muscle cells. The number of capillaries and venules per unit area of myocytes was computed. This approach was followed to eliminate the effects of variations caused by changes in the interstitial compartment. Sampling of vessel measurements involved a minimum of 20 and a maximum of 30 microscopic fields for the LV of each rat (Maestri et al., 2003).

2.9. Data analysis and statistics

Values of all parameters are expressed as mean ± SEM. Statistical analyses were carried out using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) and statistical significance for all tests was set at $p<0.05$.

In order to detect possible differences at rest between the two experimental groups, the values of R-R interval and HRV indices of 30 min baseline recordings preceding restraint and pharmacological challenges were statistically analyzed by means of two-way analysis of variance (ANOVA), with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (two levels: first

and fifth restraint test; pre- and post-IRS autonomic blockade). In order to analyze the effects of postnatal treatment on cardiac response to restraint and autonomic blockade, we calculated delta values for each 5-min time point relative to the baseline and named them delta1, delta2, ...delta9 for restraint tests, and delta1, delta2, ...delta12 for pharmacological challenges. Statistical analysis was then carried out on delta instead of absolute values to abolish possible group differences in stressor responsivity due to differences in baseline. Two-way ANOVA was applied to delta values, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (9 levels for restraint, 12 levels for pharmacological challenge).

Average light and dark phase values of RR and r-MSSD on each day before and after IRS were calculated. Control and MS values of RR and r-MSSD in baseline conditions (pre-IRS) during the light and dark phases were compared via Student's t-test. Delta values between each post-IRS day and average pre-IRS value of light and dark phases were computed. Statistical analysis on delta values was carried out by means of two-way ANOVA, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (7 days).

Following ANOVAs, posthoc analysis on ECG data was applied where appropriate using Student's t-test, after checking for variance homogeneity by means of Levene test.

Values of body weight were analyzed via two-way ANOVA for repeated measures, with "postnatal treatment" as between-subject factor (two levels: MS and control rats) and "time" as within-subject factor (6 weeks). The weight of adrenal glands at sacrifice was expressed as a ratio to the animal body weight (mg adrenal gland weight/100g body weight) and statistically compared by Student's t-test. Statistical analysis of cardiac structural measures was also carried out via Student's t-test when two groups were analyzed, or via ANOVA followed by t-test for multiple comparisons.

3. Results

3.1. Electrocardiographic response to restraint

Two-way ANOVA applied to baseline values of RR and HRV parameters measured just before the first and fifth restraint episode did not reveal significant effects of group, time, or group x time interaction. This indicates that maternal separation did not induce clear changes in resting HRV indexes, neither at the beginning (first episode) nor at the end (fifth episode) of the adult intermittent restraint period (Table I).

Table I also reports the absolute values of RR and HRV parameters for each 5-min time point during and after the first and fifth restraint episode. Two-way ANOVA on delta values (values of each 5-min time point during restraint test and recovery relative to baseline) for the first restraint test revealed only a significant effect of time for RR ($F_{8,176}$=109.5, $p<0.01$) and r-MSSD ($F_{8,176}$=9.3, $p<0.01$). No significant effect of group was observed for any of the parameters considered. Hence, MS did not affect the magnitude and temporal dynamics of the changes in sympathovagal balance recorded during and im-

Table I Values (mean ± SEM) of average R-R interval (RR), time-domain HRV parameters (r-MSSD, pNN20), frequency-domain HRV parameters (LF, HF, LF/HF) during baseline conditions (30-min average value), restraint test (15 min), and recovery phase (45 min), in maternally separated (MS; n=12) and control (n=12) rats, at restraint tests 1 and 5.

Recording period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF /HF	
		Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5	Restraint 1	Restraint 5
Baseline	MS	210.8±4.43	211.8±5.14	6.87±0.45	7.58±0.83	1.10±0.22	1.87±0.57	30.63±2.53	29.88±3.06	69.37±2.53	70.12±3.06	0.48±0.07	0.42±0.05
	control	209.9±3.45	207.7±5.90	5.84±0.68	5.50±0.77	1.19±0.32	1.06±0.54	32.88±3.38	32.04±3.35	67.12±3.38	67.96±3.35	0.57±0.10	0.56±0.09
Test													
1 (0-5 min)	MS	122.8±0.97	125.5±1.60	3.47±0.77	2.24±0.34	0.29±0.16	0.22±0.09	47.85±2.85	47.89±2.23	52.15±2.85	52.11±2.23	0.98±0.11	0.96±0.09
	control	121.2±1.08	122.1±1.23	1.69±0.17	2.07±0.22	0.12±0.08	0.10±0.05	43.01±1.84	48.06±1.61	56.99±1.84	51.94±1.61	0.77±0.06	0.90±0.07
2 (5-10)	MS	122.3±1.24	129.2±1.92	1.88±0.16	3.19±0.30	0.06±0.03	0.14±0.04	54.01±1.79	49.31±2.36	45.99±1.79	50.69±2.36	1.15±0.11	1.02±0.10
	control	123.2±2.15	131.3±2.97	2.12±0.24	2.63±0.29	0.20±0.18	0.12±0.06	51.02±1.42	49.91±2.01	48.98±1.42	50.09±2.01	1.01±0.08	0.98±0.10
3 (10-15)	MS	132.1±1.98	139.1±2.80	3.47±0.31	4.09±0.27	0.20±0.07	0.29±0.09	47.52±2.46	46.76±2.83	52.48±2.46	53.24±2.83	0.95±0.09	0.93±0.10
	control	138.0±2.24	145.4±3.95	3.15±0.37	3.45±0.52	0.22±0.07	0.36±0.20	50.17±2.33	47.29±2.01	49.83±2.33	52.71±2.01	1.06±0.11	0.80±0.09
Recovery													
4 (15-20)	MS	143.6±2.80	147.0±2.14	5.09±0.47	4.64±0.26	1.12±0.32	1.16±0.30	48.73±3.69	47.18±2.12	51.27±3.69	52.82±2.12	1.09±0.19	0.93±0.09
	control	142.6±2.60	145.4±2.16	3.66±0.42	3.75±0.42	0.55±0.20	0.50±0.16	52.67±1.67	54.88±1.79	47.33±1.67	45.12±1.79	1.14±0.07	1.15±0.13
5 (20-25)	MS	152.1±3.33	153.6±2.70	4.79±0.45	4.03±0.28	0.84±0.22	0.45±0.08	52.62±3.04	51.95±2.50	47.38±3.04	48.05±2.50	1.08±0.11	1.14±0.10
	control	146.8±2.64	152.4±1.98	3.68±0.38	3.93±0.54	0.53±0.17	0.60±0.21	53.35±2.31	54.67±2.31	46.65±2.31	45.33±2.31	1.19±0.11	1.16±0.15
6 (25-30)	MS	152.6±3.41	154.5±2.51	5.00±0.43	4.32±0.39	0.99±0.26	0.60±0.17	46.96±3.62	45.34±2.45	53.04±3.62	54.66±2.45	0.99±0.16	0.87±0.09
	control	147.1±2.92	154.9±1.52	3.51±0.24	4.03±0.62	0.37±0.10	0.57±0.25	53.44±2.20	50.73±1.14	46.56±2.20	49.27±1.14	1.09±0.15	1.00±0.06
7 (30-35)	MS	154.5±3.79	151.8±2.20	4.46±0.46	4.39±0.36	0.98±0.26	0.88±0.22	44.34±3.32	43.75±3.24	55.66±3.32	56.25±3.24	0.77±0.08	0.85±0.13
	control	151.7±1.96	154.7±3.03	4.07±0.40	3.49±0.35	1.10±0.32	0.50±0.18	50.15±1.98	46.60±2.35	49.85±1.98	53.40±2.35	1.04±0.08	0.91±0.08
8 (35-40)	MS	157.5±3.85	160.5±3.77	4.05±0.39	4.53±0.24	0.48±0.15	0.42±0.11	47.45±2.85	46.72±1.77	52.55±2.85	53.28±1.77	0.97±0.12	0.91±0.06
	control	149.7±2.30	159.8±3.66	3.64±0.43	4.19±0.51	0.54±0.25	0.55±0.22	50.57±2.71	44.33±2.02	49.43±2.71	57.67±2.02	1.09±0.13	0.82±0.06
9 (40-45)	MS	158.8±4.75	168.4±5.72	4.06±0.36	4.09±0.28	0.34±0.14	0.33±0.11	42.18±3.83	41.92±3.64	57.82±3.83	58.08±3.64	0.82±0.14	0.71±0.09
	control	158.4±3.63	174.1±5.65	4.49±0.55	4.14±0.51	0.72±0.38	0.54±0.15	43.14±3.19	41.13±3.35	56.86±3.19	58.87±3.35	0.73±0.09	0.76±0.09

Abbreviations: HF, high frequency; HRV, heart rate variability; LF, low frequency; r-MSSD, root mean square of successive differences between adjacent R-R intervals; n.u., normalized units; pNN20, percentage of successive interval differences larger than 20 ms.

mediately after an acute stressor in adulthood. At the fifth (last) restraint test, the time course of RR and HRV parameters was again similar in MS and control rats (Table I). Two-way ANOVA on delta values revealed a significant effect of time for RR ($F_{8,176}$=91.9, $p<0.01$), r-MSSD ($F_{8,176}$=16.6, $p<0.01$), LF ($F_{8,176}$=5.2, $p<0.05$), HF ($F_{8,176}$=5.2, $p<0.05$), and LF/HF ($F_{8,176}$=7.3, $p<0.05$). However, a significant effect of postnatal treatment was found only for r-MSSD ($F_{1,22}$=5.1, $p<0.05$). Posthoc analysis on r-MSSD delta values revealed only sporadic differences between the two experimental groups, which were limited to a few time points in the recovery phase ($t_{delta5;(22)}$= 2.06, $p<0.05$; $t_{delta6;(22)}$ = 2.32, $p < 0.05$; $t_{delta8;(22)}$= 2.61, $p < 0.01$; $t_{delta9;(22)}$= 2.60, $p<0.05$).

3.2. Electrocardiographic response to pharmacological autonomic blockade

Two-way ANOVA applied to baseline values of RR and HRV parameters did not reveal any significant effect of group, time, or group x time interaction. MS did not induce changes in resting HRV indices, neither at pre-IRS nor at post-IRS pharmacological autonomic challenge (Table II).

Table II also reports the absolute values of RR and HRV parameters for each 5-min time point during the first and second pharmacological challenge. Two-way ANOVA on delta values applied to the first pharmacological challenge revealed a significant effect of time for RR ($F_{11,242}$=401.6, $p<0.01$), r-MSSD ($F_{11,242}$=7.9, $p<0.05$), LF ($F_{11,242}$=21.7, $p<0.01$), HF ($F_{11,242}$=21.7, $p<0.01$), and LF/HF ($F_{11,242}$=16.8, $p<0.01$). When applied to the second pharmacological challenge, two-way ANOVA on delta values revealed a significant effect of time for RR ($F_{11,242}$=915.2, $p<0.01$), LF ($F_{11,242}$=14.1, $p<0.01$), HF ($F_{11,242}$=14.2 $p<0.01$), and LF/HF ($F_{11,242}$=14.9, $p<0.01$). However, significant effects of group were not observed; hence, MS did not affect cardiac autonomic responsiveness to vagal and sympathetic blockade, neither before nor after IRS.

3.3. Daily rhythms in cardiac activity

MS and control rats exhibited similar light and dark phase values of heart rate (RR) and vagal activity (r-MSSD) under baseline conditions, i.e. before the IRS stress period (RR_{light}: t_{22}= 0.72, p = n.s.; RR_{dark}: t_{22}= 1.20, p = n.s.; r-$MSSD_{light}$: t_{22}= 0.51, p = n.s.; r-$MSSD_{dark}$: t_{22}= 1.45, p = n.s.; Figure 2A,C).

Two-way ANOVA on RR delta values (value at each post-IRS day relative to the pre-IRS reference value; Figure 2B) did not reveal any significant effect of group, time or group x time interaction, neither for the light nor for the dark phase. Hence, MS did not affect daily heart rate rhythmicity, neither before nor after IRS.

However, two-way ANOVA on delta values of r-MSSD (Figure 2D) revealed a significant effect of postnatal treatment for both the light and dark phase of the daily rhythm (r-$MSSD_{light}$: group $F_{1,22}$=10.8, $p<0.01$; r-$MSSD_{dark}$: group $F_{1,22}$=36.5, $p<0.01$). Control rats exhibited higher delta values of r-MSSD in the active (dark) and passive (light) phase of the circadian rhythm, with significant differences compared to MS counterparts

Table II Values (mean ± SEM) of average R-R interval (RR), time-domain (r-MSSD, pNN20) and frequency-domain HRV parameters (LF, HF, LF/HF) during baseline conditions (30-min average value) and after scopolamine (15 min) and atenolol (45 min) injections, in maternally separated (MS; n=12) and control (n=12) rats, at pharmacological challenges 1 and 2.

Recording period	Group	RR (ms)		r-MSSD (ms)		pNN20 (%)		LF (n.u.)		HF (n.u.)		LF/HF	
		challenge 1	challenge 2	challenge 1	challenge 2	challenge 1	challenge 2	challenge 1	challenge 2	challenge 1	challenge 2	challenge 1	challenge 2
Baseline	MS	203.6±3.89	205.9±5.41	6.47±0.70	6.01±0.61	1.39±0.38	1.72±0.57	36.29±3.50	31.55±2.35	63.71±3.50	68.45±2.35	0.57±0.07	0.50±0.06
	control	205.3±4.23	200.7±5.76	5.38±0.56	5.60±0.57	0.93±0.26	1.57±0.44	36.86±1.96	38.78±1.83	63.14±1.96	61.22±1.83	0.61±0.05	0.64±0.07
Scopolamine													
1 (0-5 min)	MS	136.8±2.51	138.5±1.09	1.47±0.17	1.69±0.16	0.09±0.04	0.05±0.02	47.28±2.39	48.34±2.62	52.72±2.39	51.66±2.62	0.94±0.09	0.99±0.11
	control	136.7±3.65	137.1±2.39	1.66±0.20	1.73±0.19	0.06±0.02	0.08±0.04	50.28±1.95	48.36±3.26	49.72±1.95	51.64±3.26	1.05±0.08	1.01±0.11
2 (5-10)	MS	133.5±2.14	134.6±2.05	0.78±0.06	0.87±0.07	0.00±0.00	0.00±0.00	48.48±2.02	46.36±2.21	51.52±2.02	53.64±2.21	0.98±0.08	0.89±0.08
	control	135.8±3.26	131.6±2.22	0.90±0.08	1.10±0.13	0.01±0.08	0.04±0.02	49.05±1.86	54.45±2.94	50.95±1.86	45.55±2.94	0.94±0.08	1.29±0.13
3 (10-15)	MS	131.9±1.70	135.8±2.52	0.99±0.14	0.76±0.06	0.01±0.01	0.00±0.00	48.69±3.29	48.49±3.73	51.31±3.29	51.51±3.73	1.03±0.12	0.94±0.12
	control	137.4±3.15	133.5±2.75	0.96±0.16	0.95±0.12	0.05±0.04	0.00±0.00	53.95±2.32	58.59±1.67	46.05±2.32	41.41±1.67	1.16±0.13	1.37±0.11
Atenolol													
4 (15-20)	MS	155.2±1.42	154.7±1.28	1.79±0.24	2.76±0.45	0.28±0.09	0.50±0.19	54.32±2.45	55.67±2.34	45.68±2.45	44.33±2.34	1.18±0.12	1.32±0.12
	control	155.2±2.22	155.6±1.77	1.93±0.34	1.72±0.19	0.26±0.12	0.12±0.03	48.24±2.90	50.23±3.27	51.76±2.90	49.77±3.27	1.00±0.11	1.01±0.12
5 (20-25)	MS	174.8±2.69	173.6±1.12	1.22±0.14	1.57±0.15	0.03±0.02	0.02±0.01	46.61±1.56	42.17±3.42	53.39±1.56	57.83±3.42	0.84±0.07	0.72±0.08
	control	177.2±2.26	174.0±2.65	1.10±0.07	1.15±0.09	0.01±0.01	0.00±0.00	41.01±2.30	42.91±4.25	58.99±2.30	57.09±4.25	0.72±0.06	0.84±0.12
6 (25-30)	MS	185.5±2.09	181.4±1.87	1.25±0.14	1.48±0.15	0.04±0.02	0.04±0.04	46.43±2.77	38.13±3.16	53.57±2.77	61.87±3.16	0.91±0.09	0.66±0.08
	control	185.2±2.55	180.1±2.86	1.38±0.09	1.50±0.15	0.02±0.01	0.01±0.01	38.28±1.94	39.88±4.84	61.72±1.94	60.12±4.84	0.64±0.05	0.68±0.11
7 (30-35)	MS	189.6±2.62	185.5±2.31	1.82±0.30	1.60±0.16	0.10±0.05	0.08±0.04	36.43±3.86	37.53±3.76	63.57±3.86	62.47±3.76	0.56±0.07	0.66±0.10
	control	185.8±1.68	182.1±3.24	2.19±0.21	1.66±0.20	0.14±0.05	0.07±0.06	44.25±3.22	42.95±3.54	55.75±3.22	57.05±3.54	0.86±0.11	0.76±0.12
8 (35-40)	MS	191.0±2.73	186.9±2.42	1.65±0.28	1.52±0.13	0.07±0.04	0.00±0.00	41.67±4.27	38.89±4.01	58.33±4.27	61.11±4.01	0.83±0.15	0.63±0.08
	control	187.5±1.49	185.9±3.22	1.50±0.17	1.54±0.16	0.04±0.02	0.02±0.01	33.21±3.80	36.98±2.52	66.79±3.80	63.02±2.52	0.49±0.07	0.61±0.05
9 (40-45)	MS	193.3±2.63	190.1±2.75	1.88±0.24	1.42±0.20	0.05±0.02	0.01±0.01	38.30±4.36	33.74±3.42	61.70±4.36	66.26±3.42	0.69±0.10	0.55±0.08
	control	189.6±2.20	188.2±3.05	1.37±0.11	1.34±0.16	0.01±0.01	0.00±0.00	38.46±2.59	35.26±2.74	61.54±2.59	64.74±2.74	0.61±0.07	0.58±0.07
10 (45-50)	MS	196.5±3.20	191.8±2.39	1.68±0.17	1.32±0.14	0.09±0.06	0.03±0.03	45.18±1.56	32.19±5.57	54.82±1.56	67.81±5.57	0.78±0.08	0.48±0.11
	control	191.7±3.13	189.9±3.22	1.44±0.12	1.31±0.11	0.08±0.04	0.03±0.02	41.89±2.75	37.24±5.12	58.11±2.75	62.76±5.12	0.64±0.08	0.73±0.17
11 (50-55)	MS	194.9±2.93	193.6±2.27	2.38±0.23	1.29±0.11	0.12±0.04	0.01±0.01	42.70±5.19	31.07±4.93	57.30±5.19	68.93±4.93	0.86±0.13	0.55±0.13
	control	193.5±3.53	189.5±3.58	1.46±0.14	1.37±0.17	0.04±0.02	0.00±0.00	38.57±3.61	42.77±5.54	61.43±3.61	57.23±5.54	0.60±0.08	0.79±0.15
12 (55-60)	MS	196.7±2.65	195.4±2.76	2.10±0.28	1.37±0.10	0.11±0.05	0.00±0.00	34.98±4.35	34.00±5.00	65.02±4.35	66.00±5.00	0.70±0.10	0.60±0.12
	control	196.9±3.04	189.2±3.77	1.27±0.16	1.09±0.14	0.00±0.00	0.00±0.00	29.37±3.42	39.00±5.22	70.63±3.42	61.00±5.22	0.46±0.08	0.82±0.19

Abbreviations: HF, high frequency; HRV, heart rate variability; LF, low frequency; r-MSSD, root mean square of successive differences between adjacent R-R intervals; n.u., normalized units; pNN20, percentage of successive interval differences larger than 20 ms.

on all days (1-7) following IRS for the dark phase ($2.6 \leq t_{22} \leq 4.6$, $p<0.05$) and on days 1-4 and 6 following IRS for the light phase ($2.7 \leq t_{22} \leq 4.4$, $p<0.05$) (Figure 2D).

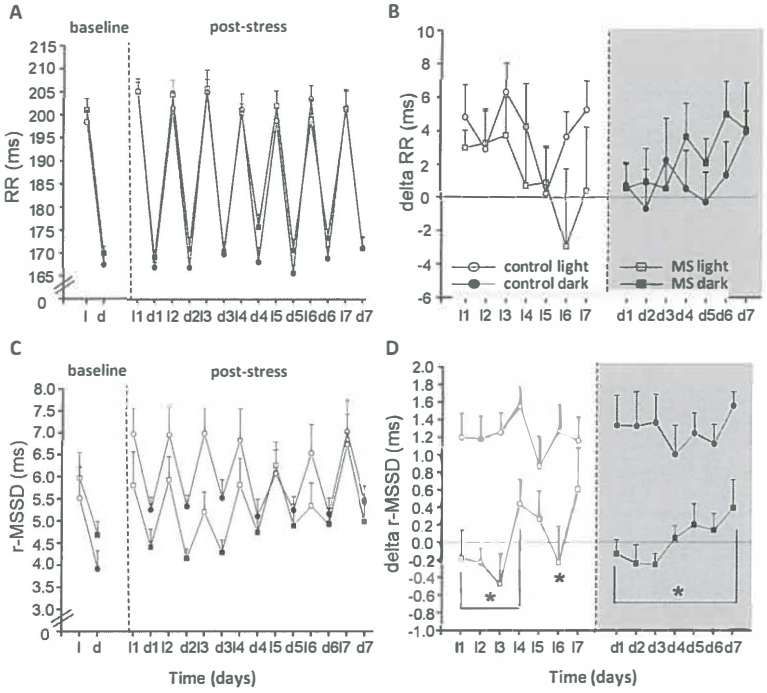

Figure 2 *Daily rhythmicity of heart rate before and after the intermittent restraint protocol, in maternally separated and control rats. Average RR values (panel A) and r-MSSD values (panel C) for the 12h light (open symbols) and dark phases (solid symbols) before (baseline: l and d) and after (post-stress: l1-l7 and d1-d7) IRS. Delta values between each post-IRS day and the average pre-IRS value of light and dark phases are also reported (panel B and D), for both control (n=12) and maternally separated (MS, n=12) rats. R-R, average R-R interval duration (ms). r-MSSD, root mean square of successive differences between adjacent R-R intervals (ms). Data are mean ± SEM. *p < 0.05, significant differences between MS and control animals (Student's t-test).*

3.4. Body weight and adrenal gland weight

Body weight values were similar in control and MS rats at the time of transmitter implantation (466 ± 11 vs. 462 ± 13 g) and euthanasia (457 ± 9 and 460 ± 11g). Two-way ANOVA on body weight values revealed a significant effect of time ($F_{5,110}=8.44$, $p<0.01$) due to post-surgery weight loss, which was similar in the two groups of rats. However, no differences were found between MS and control rats across the experimental protocol. Hence, MS did not produce changes in body weight temporal dynamics, which were similar in control and MS rats both before and after IRS. However, MS rats at euthanasia had significantly heavier adrenals compared to controls (MS: 20.99 ± 1.18 mg/100g; control:

16.50 ± 1.27 mg/100g) (t_{22}=2.60, $p<0.05$). In addition, adrenals excised from 10 additional animals exposed only to early life manipulation were significantly larger than those of controls (n=10; MS: 17.58 ± 1.46 mg/100g; control: 13.38 ± 1.13 mg/100g; t_{18}=2.27, $p<0.05$).

Table III Values (mean ± SEM) of gross cardiac characteristics, myocardial fibrosis in the LV and vascular distribution in the LV, in maternally separated (MS; n=12) and control (n=12) rats.

	control	MS
LVW (mg)	889.5 ± 22.08	818.8 ± 20.17 *
RVW (mg)	201.9 ± 7.01	201.8 ± 8.11
LVW/HW (mg/mg)	0.908 ± 0.005	0.802 ± 0.006 *
RVW/HW (mg/mg)	0.192 ± 0.005	0.198 ± 0.006
LV chamber length (mm)	14.58 ± 0.27	14.20 ± 0.23
LV chamber equatorial diameter (mm)	5.32 ± 0.18	5.02 ± 0.15
LV wall thickness (mm)	2.11 ± 0.06	2.14 ± 0.06
LV chamber volume (mm^3)	220 ± 15.67	189 ± 11.90 *
Perivascular fibrosis (%)	0.316 ± 0.047	0.225 ± 0.043 *
Interstitial fibrosis (%)	0.158 ± 0.060	0.243 ± 0.056 *
Capillary density (n/mm^2)	132.2 ± 16.61	178.8 ± 11.42 *
Venule density (n/mm^2)	6.31 ± 1.02	9.13 ± 2.54

Abbreviations: LV, left ventricle; LVW, left ventricular weight; RVW, right ventricular weight; HW, heart weight; *p<0.05: significant differences between MS and control rats (Student's t-test).

3.5. Cardiac anatomy and myocardial structure

As shown in Table III, slight differences were observed between MS and control rats with respect to LVW and LV linear parameters. Actual LVW and weight relative to HW were significantly lower in the MS group, whereas RVW was similar in MS and control rats (Table III). As a result of modest reductions in both LV length (-3%) and equatorial diameter (-6%), chamber volume was significantly smaller (-14%) in MS rats compared to control rats (Table III). To determine whether these changes in gross cardiac anatomy were accompanied by changes in cardiomyocyte dimensions, myocyte cross sectional area (CSA) was measured. In the MS group, cardiomyocyte CSA was 5% larger than in the controls (294.09 ± 3.92 vs. 278.79 ± 2.28 μm^2; t_{22}=2.34, $p<0.05$), indicating that MS induced a mild hypertrophy of individual cells.

The morphometric analysis of the myocardium documented no significant differences between the two groups of rats with respect to total amount of myocardial fibrosis in the LV myocardium (MS: 0.468 ± 0.077 % vs. control: 0.474 ± 0.080 % n.s.). The volume fraction of myocytes was also unaffected by the experimental conditions (MS: 91.41 ± 0.55 % vs. control: 91.38 ± 0.70 %, n.s). Perivascular and interstitial fibrosis, including small foci of collagen accumulation distributed in the myocardium, were present in both MS and control hearts. Interestingly, these forms of collagen accumulation were differently expressed in the experimental rats. Interstitial fibrosis was slightly larger in MS rats when the ventricular wall was considered as a whole (Table III) and also when the three different layers where separately examined, i.e. endomyocardium, midmyocardium,

and epimyocardium (MS: 0.12 ± 0.06 %, 0.42 ± 0.16 % and 0.18 ± 0.07 %, respectively) compared to control group (0.02 ± 0.02 %, 0.35 ± 0.12 and 0.10 ± 0.04 %, respectively). Perivascular fibrosis was more abundant in the midmyocardium and epimyocardium of control hearts (0.29 ± 0.10 % and 0.76 ± 0.14 % vs. MS: 0.19 ± 0.06 % and 0.44 ± 0.09 %, respectively), resulting in an overall larger amount of this form of collagen deposition in these rats (Table III).

The morphometric evaluation of vascular structures in the LV myocardium (Table III) showed that capillary density was significantly larger in MS rats compared to control counterparts. Although venule density was increased 1.5-fold in MS rats, this value did not reach statistical significance (Table III).

4. Discussion

The aims of this study were to investigate the long-term effects of early life adverse experience on (i) autonomic neural regulation of heart rate and (ii) cardiac morphological/morphometrical characteristics in male rats.

The results obtained indicate that MS occurring in the first 2 weeks of neonatal life did not substantially alter adult cardiac sympathovagal balance, neither at rest nor under acute challenging conditions, namely repeated restraint stress and pharmacological autonomic blockade. However, MS rats did not show the enduring vagal rebound exhibited by control counterparts in the days following adult intermittent stress, which involved both the night and light phases of the circadian rhythm. In addition, MS induced a few minor changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage.

In this study, early MS was combined with a stress protocol based on intermittent restraint episodes in adulthood. MS did not change in a significant manner the acute response to a single restraint test and the habituation profile of cardiac autonomic response through repeated restraint episodes. Interestingly, in an experimental procedure resembling the present one (i.e. a 2-week daily MS followed by IRS in adulthood), Hulshof and colleagues (2011) reported a similar lack of differences between control and MS rats in HPA axis stress reactivity and habituation (i.e. similar plasma adrenocorticotrophic hormone and corticosterone levels).

In this paper, cardiac autonomic balance was assessed by means of a large number of heart variability indices, belonging to both time and frequency domains. During restraint test 1 and 5, MS and control rats exhibited a similar increase in cardiac chronotropism and an incomplete return to baseline heart rate values during the immediate post-test period. Concomitant, reduced values of time-domain HRV indices and HF power point to a withdrawal of the parasympathetic drive to the heart (Kleiger et al., 1993), which did not recover to initial values until the end of the test. Accordingly, the increase in LF power and LF/HF ratio during both restraint and recovery period points to a shift of autonomic regulation toward a sympathetic prevalence (Aubert et al., 1999). Notably, these HRV data also indicate that the dynamics of cardiac autonomic balance were similar across restraint episodes in MS and control rats, i.e. both groups did not exhibit habituation-like effects.

As expected (Japundzic et al., 1990; Aubert et al., 1999), the injection of a cholinergic muscarinic blocker (methylscopolamine) induced a robust increase of heart rate, accompanied by a clear reduction in the values of r-MSSD and pNN20. The decrease in HF power was accompanied by an increase in LF power and LF/HF ratio. Subsequent injection of a beta-blocker (atenolol) caused the return of heart rate and spectral indices of HRV to their basal values, whereas time-domain parameters remained substantially suppressed. Overall, the results obtained with the double pharmacological autonomic blockade indicate that MS had no substantial effects on cardiac pacemaker intrinsic activity, neither before nor after IRS.

So far few studies have investigated the long-lasting consequences of MS on cardiovascular function and, to our knowledge, the present work is the first to explore long-term cardiac autonomic effects through HRV parameters. The lack of differences between MS and control rats, both at rest and during acute environmental and pharmacological challenge, supports the idea that MS per se does not bring about a cardiac autonomic pathophysiologic phenotype. In this regard, our findings are in agreement with a number of other studies that did not find any clear effect of MS on adult baseline heart rate and arterial blood pressure (Sanders and Anticevic, 2007; Loria et al., 2010a,b). However, in the study by Sanders and Anticevic (2007) on borderline hypertensive rats, MS rats showed higher heart rate responsivity to restraint stress compared to control rats, despite blood pressure response was similar in the two groups. Although the MS protocol in the latter study was similar to ours, it may well be that borderline hypertensive rats are more susceptible to environmental stressors and that different blood pressure phenotypes play a pivotal role in mediating the differences in autonomic regulation of cardiac stress response.

The recording of the daily rhythms of heart rate and cardiac vagal activity allowed further exploration of the long-lasting effects of MS on cardiac chronotropy and its autonomic neural regulation, before and after the period of adult intermittent stress. MS and adult exposure to five restraint episodes did not affect the light-dark oscillation of heart rate. However, while autonomic control of cardiac chronotropy remained largely unchanged in MS rats, control counterparts showed a prolonged increase in cardiac parasympathetic drive following IRS, involving both the dark and light phases of the circadian rhythm. As the average night and day values of heart rate were similar before and after the stress period, we hypothesize that the increase of parasympathetic drive observed in control rats following IRS was accompanied by a concomitant enhancement of sympathetic tone.

This peculiar phenomenon, which was observed in control but not in maternally separated rats, might be called "enduring vagal rebound" or "persistent vagal rebound". In the literature, "vagal rebound" usually has a rather different meaning; it refers to short-term, brief vagal hyperactivity following a stressor, a sympathetic overdrive or reperfusion after acute myocardial infarction (Chiladakis et al., 2001; Mezzacappa et al., 2001). In this paper, it was a relatively persistent, long-term consequence of an intermittent stressor and can be viewed as an adaptive response of the organism. Indeed, the increase in vagal activity that often occurs to counterbalance stress-induced sympathetic activation is known

to be associated with a reduced risk of cardiovascular disease and mortality (La Rovere et al., 1998).

If this point of view is correct, one may infer that cardiac autonomic neural control of adult rats that experienced early life challenge was less stress-responsive (i.e. perhaps less flexible) compared to control counterparts, and thus less favorable in terms of resilience. However, one may also maintain that the lack of changes in autonomic modulation observed in MS rats was the consequence of a shift of the regulatory range, which implied a reduced sensitivity to adult intermittent stress exposure (Koolhaas et al., 2011). Indeed, several studies support the view that early maternal environment prepares the regulatory range of the offspring for the conditions it may have to cope with in adulthood (Kaiser and Sachser, 2005; Gluckman et al., 2007; Champagne et al., 2008). An example of this is given in a recent study in mice by Heiming and co-workers (2009): they showed that mice that were raised in a threatening environment exhibited less anxiety and more exploratory behavior as adults when confronted with challenging situations than animals that were raised in a stable environment.

One may question that the data collected following scopolamine administration do not support the observed raise in vagal activity emerging from post-stress rhythm analysis. Indeed, one may expect a somewhat larger tachycardic response to scopolamine in control animals after IRS. The likely reason for this discrepancy is that the second pharmacological challenge was carried out during the light phase of the day after the end of rhythm recordings (day 31 in Figure 1). Figure 2 (panel D) shows that r-MSSD values of control rats in the last light phase (l7) were not significantly different anymore from corresponding MS values. Hence, the pharmacological challenge was carried out when the "vagal difference" between control and MS rats was already gone.

In the current study, animals exposed to MS and adult intermittent stress had heavier adrenal glands compared to control rats, which may reflect either enhanced steroidogenesis in the cortex or an increased catecholamine biosynthetic activity in the medulla (or both). However, increased adrenal weight in MS rats that were not exposed to adult manipulations suggests that MS per se is able to induce adrenal enlargement. Indeed, available data from the literature on the effects of postnatal stress on adrenal gland weight are not consistent. Some authors showed that 24h maternal deprivation induces significantly heavier adrenals (Rots et al., 1996; Husum et al., 2002), while others reported no adrenal enlargement (Wigger and Neumann, 1999) or even lighter glands (Slotten et al., 2006). We did not determine plasma corticosterone concentrations in this study, so we cannot relate any cardiac changes to increased corticosterone secretion.

The evaluation of the effects of MS on cardiac architecture indicated a moderate reduction in LV weight and chamber dimensions in the absence of clear changes in myocardial structural composition. Myocardial fibrosis was minimally affected by MS and only interstitial deposition of collagen was moderately increased. These gross anatomical changes, in association with modest signs of cellular hypertrophy, likely reflect a physiological adaptation to maternal stress of the growing heart during early postnatal development. The possibility cannot be excluded that a stressor imposed during a delicate phase of cardiac development in which remarkable cellular changes occur may structurally

affect the heart. It is well established that a switch from a prevailing hyperplastic to a predominant hypertrophic growth (Anversa et al., 1980) as well as apoptotic death of cardiomyocytes (Kajstura et al., 1995) all rapidly interplay within 3 weeks after birth in the rat heart. Whether MS-driven neurohormonal factors, implicated in physiologic and pathologic processes in the heart, may alter cardiomyocyte turnover resulting in cardiac structural remodeling following maturation is unknown. Slight hypertrophy of cardiomyocytes was observed here in the LV in association with a reduced mass and modest collagen deposition, indicating that precocious cell loss and reactive hypertrophy may have occurred early during postnatal cardiac development. Interestingly, all these events are known to be evoked in the heart by the renin-angiotensin system (Sadoshima and Izumo, 1993), which in turn has been implicated in MS (Loria et al., 2010b).

The early postnatal period is also characterized by a dramatic growth of the coronary vascular bed and the functional capacity of the myocardium reaches adult values at approximately 16 days of age (Hopkins et al., 1973). The mean transmural number of capillaries undergoes a fourfold increase in the LV from postnatal day 1-11 and the aggregate ventricular capillary length more than doubles between postnatal day 5 and 11 (Olivetti et al., 1980). Our data point to an increased capillarization of the left ventricular myocardium in maternally separated animals. The detected increase in the density of vascular structures is relevant under the contention that enlargement of cardiomyocytes, as shown in the myocardium of MS rats, would result in the reduction in the number of capillaries per unit area (n/mm^2) according to morphometric principles. However, why promotion of angiogenesis or lack of regression of capillary formation should occur in the heart under these experimental conditions requires further investigation.

In conclusion, this study on rats shows that daily 3h MS during the first 2 weeks of postnatal life did not affect in a significant manner adult intrinsic heart rate and acute cardiac autonomic responsivity to a psychological stressor. However, maternally separated rats did not exhibit the enduring vagal rebound observed in control counterparts after adult IRS, which might be viewed as an adaptive response aiming at favoring cardiovascular stress resilience. Moreover, MS induced some, although minor, changes in cardiac anatomy, involving modest degrees of cardiomyocyte hypertrophy, increased angiogenesis, and myocardial damage. Whether these functional and structural changes could have evolved to a more pathologic cardiac phenotype later in life is plausible, but remains to be determined.

The overall mildness of cardiac functional and structural effects of MS reported in this paper indicates that this approach might not be the best preclinical tool for modeling early-age, stress-induced cardiac disease in humans.

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3.

Maternal separation decreases adult hippocampal cell proliferation and impairs cognitive performance but has little effect on stress sensitivity and anxiety in adult Wistar rats

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Marcus Miller - Tomaas (Tutu revisited)

Abstract

Stressful events during childhood are thought to increase the risk for the development of adult psychopathology. A widely used animal model for early life stress is maternal separation (MS), which is thought to affect development and cause alterations in neuroendocrine stress reactivity and emotionality lasting into adulthood. However, results obtained with this paradigm are inconsistent. Here we investigated whether this variation may be related to the type of stressor or the tests used to assess adult stress sensitivity and behavioral performance. Rat pups were exposed to a 3h daily MS protocol during postnatal weeks 1-2. In adulthood, animals were subjected a wide variety of stressors and tests to obtain a better view on the effects of MS on adult hypothalamic-pituitary-adrenal (HPA) axis regulation, anxiety-like behavior, social interaction and cognition. Also, the influence of MS on adult hippocampal neurogenesis was studied because it might underlie changes in neuroendocrine regulation and behavioral performance. The results show that, independent of the nature of the stressor, MS did not affect the neuroendocrine response. MS did not influence anxiety-like behavior, explorative behavior and social interaction, but did affect cognitive function in an object recognition task. The amount of new born cells in the hippocampal dentate gyrus was significantly decreased in MS animals; yet, cell differentiation and survival were not altered. In conclusion, while interfering with the mother-infant relationship early in life did affect some aspects of adult neuroplasticity and cognitive function, it did not lead to permanent changes in stress sensitivity and emotionality.

1. Introduction

Epidemiological studies show that adverse early life events are associated with altered stress responsiveness and enhanced vulnerability to the development of psychopathologies like depression and anxiety disorders (Heim and Nemeroff, 2001). Animal models of early life stress commonly aim at the postnatal disruption of the mother-infant interaction. The two most well known models applied in laboratory rats are early handling (EH) and maternal separation (MS). EH consists of brief (3-15 min) daily separations of mother and offspring during the first postnatal weeks and rather consistently reduces the neuroendocrine stress response and decreases the level of anxiety during adulthood (for review, see Lehmann and Feldon, 2000). MS on the other hand originally consisted of separating mother and offspring for a more prolonged period of 3 hours per day during the first weeks after birth (Plotsky & Meaney, 1993). Currently, also other MS paradigms are used, in which duration and frequency, but also postnatal period in which MS is carried out differ. However, in the present paper we applied and focussed on the MS model as originally developed by Plotsky & Meaney (1993).

While it is a generally accepted idea that in comparison to undisturbed controls, MS permanently changes the offspring's neuroendocrine and behavioral stress reactivity in an opposite direction as EH, the actual data are far from consistent (for review, see Lehmann and Feldon, 2000). MS was found to affect the adult HPA-axis regulation, as shown by changes in both basal activity and altered stress reactivity (Daniels et al., 2004; Marais et al., 2008; Plotsky & Meaney, 1993; Wigger et al., 1999). While some studies show an increase in the adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels in maternally separated rats after stress (Aisa et al., 2007; Huot et al., 2002; Plotsky et al., 2005), others report no significant differences between MS and non-handled control groups (Mirescu et al., 2004; Plotsky & Meaney, 1993; Slotten et al., 2006; Wigger et al., 1999) or even lower levels in MS animals (Daniels et al., 2004; Marais et al., 2008). A similar inconsistency as with the HPA data can be found in the effects of MS on adult anxiety-like behavior and cognition. Several studies showed an increase in anxiety-like behavior in different anxiety tests (Aisa et al., 2007; Huot et al., 2002; Wigger et al., 1999), while other studies did not (de Jongh et al., 2005; Slotten et al., 2006). Even after a twice daily 3 hour separation paradigm, maternally separated animals were not found to be more anxious in adulthood (Eklund et al., 2006). In fact, in a few cases MS was reported to decrease anxiety-like behavior (Maniam et al., 2010). Also, maternally separated animals showed a more passive (stress) coping style (Gardner et al., 2005; Veenema et al., 2007), increased aggression (Veenema et al., 2007) and anhedonic behavior (Aisa et al., 2007) in some studies, while these behaviors remained unaffected in others (Marais et al., 2008; Shalev et al., 2002; Wigger et al., 1999). Furthermore, adult cognitive function in MS rats as measured by Morris water maze learning and novel object recognition, was shown to be either impaired (Aisa et al., 2007) or unaffected (Grace et al., 2009).

The hippocampus is a structure involved in HPA-axis regulation and cognitive function that still goes through a stage of dynamic development during early postnatal life (Herman & Mueller, 2006; Jarrard, 1993). For example, granule cell neurogenesis in the

dentate gyrus, which starts during late embryogenesis, continues during the first postnatal weeks (Altman & Bayer, 1990b). MS during this vulnerable postnatal period might interfere with the normal development of the hippocampus and thereby hippocampal functioning later in life (Tanapat et al., 2001). Indeed, adult cell proliferation was found to be decreased in maternally separated animals in some studies (Aisa et al., 2009; Mirescu et al., 2004); yet, it remained unaltered in other studies (Nair et al., 2007).

In this study, we evaluated the hypothesis that the substantial variation in the outcome of MS may be related to the variation in tests and paradigms used to asses stress sensitivity and behavioral performance in adulthood. Changes in adult stress sensitivity might only become apparent under certain conditions. Therefore, in the current study we first investigated whether the absence or presence of differences in neuroendocrine responses between MS and control rats is related to the nature and severity of the stressor applied in adulthood. In order to obtain a complete picture of the effects of MS on adult HPA-axis regulation and reactivity, we measured the neuroendocrine response to a wide range of stressors, from mild to more severe, emotional as well as physical.

Then to test whether the inconsistent outcome of MS in terms of behavioral responses might be related to the paradigms and testing conditions as well, we examined the effects of MS alone and in combination with adult stress exposure in different complementary behavioral paradigms, focusing on anxiety-like and explorative behavior, as well as cognitive function. Finally, since changes in adult behavior and cognitive function in MS rats might be partly related to changes in hippocampal function and the way it is affected by stress, we examined the effects of MS alone and in combination with adult stress exposure on different aspects of adult hippocampal neurogenesis.

2. Method

2.1 Animals and maternal separation paradigm

The study was performed with Wistar rats. All animals were housed under a 12h/12h light-dark cycle with temperature maintained at 21 ±1 °C. Food and water was available ad libitum. The experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen. Female and male Wistar rats were paired for a period of ten days, where after the males were removed. Pregnant females were left undisturbed, except for a daily visual check for the presence of pups. On the day of birth (postnatal day 1), the nests were culled to 10 pups, with a fifty-fifty male/female ratio where possible. Whole litters were randomly assigned to one of two groups: maternal separation (MS) and control (C). The control nests were left undisturbed until weaning, except for cage cleaning at postnatal day 10. The maternal separation nests were subjected to a procedure that previously was found to alter adult neuroendocrine and behavioral reactivity (Plotsky & Meaney, 1993). MS consisted of daily separation of the litter and the dam for 3h (9:00-12:00) from postnatal day 2 until 15. During these 3h periods whole litters were transferred to another room, to prevent vocal communication between mother and pups. The pups were placed in glass beakers lined with paper towel, which were then placed in a water bath

maintained at a temperature of 32-33°C, consistent with normal nest temperature (Schmidt et al., 1986). By this a drop in the pups' body temperature is prevented, which was shown to increase maternal care upon reunion (Leon et al., 1978; Stern & Johnson, 1990). High levels of maternal care were found to reduce behavioral and neuroendocrine stress reactivity and enhance learning and memory (Francis et al., 1999; Liu et al., 1997; Liu et al., 2000) and might therefore attenuate or cancel out the effects of maternal separation. After the 3h separation period, the pups were returned to their home cage, followed by the mother. Like the controls nests, cages of the maternally separated litters were cleaned at postnatal day 10. On postnatal day 21, all litters were weaned and the young animals remained together with their same-sex littermates under standard housing conditions until an adult age of approximately 2.5 months. During adulthood two different experiments were carried out. All adult treatment groups consisted of 8 animals.

2.2 Experiment 1: HPA axis (re)activity

The first experiment was aimed at assessing the adult HPA-axis reactivity of MS and C rats under a range of different conditions. During adulthood, the males of both treatment groups were individually housed and subjected to various stressors, from mild to more severe and to physical as well as emotional stressors. In all cases, the animals were exposed to the different stressors for a fixed period of 30 minutes during the first hours of the light phase, when basal activity of the HPA axis is at its lowest.

2.2.1 Stress paradigms

Novelty To assess the HPA-axis response to a mild challenge, animals were exposed to a novel environment. The animals were transported to a testing chamber where they were placed in a new but empty standard cage without bedding material.
Fox odor To determine the effect of a natural and purely emotional stressor on stress reactivity the animals were exposed to 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Phero Tech Inc., Vancouver, Canada), which is the main component of fox faeces and is known to provoke a HPA-axis response in rodents (Vernet-Maury et al., 1984). The day after novelty exposure the animals were transferred to the same testing room and empty cages, to prevent novelty effects, where after a vial containing gauze and 75 µl of TMT was placed in the middle of the cage.
Fear conditioning One week after the exposure to fox odor, rats were subjected to a fear conditioning paradigm, which consists of a footshock session (a severe physical stressor), and a re-exposure to the footshock box 24 hours later, during which the animals did not receive further shocks (a purely emotional stressor). During the footshock session the animals received 5 shocks, each of a duration of 3 seconds and an intensity of 0.8 mA.
Restraint stress and stress habituation A week after the fear conditioning paradigm, we examined if C and MS rats would not only respond differently to a stressor but also habituate differently following repeated exposure to this stressor. Rats were subjected to immobilization or restraint stress by enclosing them in a wire mesh restrainer fitted closely

to body size for 30 minutes daily on 5 consecutive days. It was shown previously that such repeated exposure to restraint leads to rapid habituation and decrease of the HPA axis response (Ma et al., 1998).

2.2.2 Blood sampling and analysis

For each of the stress conditions mentioned, blood samples were taken at three time points: prior to the stress exposure (baseline), directly after the 30 minutes stress exposure (peak HPA axis response) and 45 minutes after the stressor (recovery of the stress response). For the habituation stress paradigm blood was sampled only on day 1 and day 5. Blood samples of approx. 0.5 ml were drawn from the tail (Fluttert et al., 2000; Meerlo et al., 2002; Vahl et al., 2005) and collected in prechilled cups containing EDTA as anticoagulant. Thereafter, the samples were centrifuged at 4°C for 15 minutes at 2600 g, and the supernatant was stored at -80°C until further analysis. ACTH and CORT levels were determined by radioimmunoassays (MP Biomedicals, Orangeburg, NY, USA).

2.3 Experiment 2: Behavior and Neurogenesis

The aim of the second experiment was to assess whether MS and control animals differ in anxiety-like and explorative behavior, cognition and neurogenesis in adulthood. Since MS is thought to sensitize animals for succeeding stressful events, we also examined the effects of MS in combination with an adult stressor on behavior and neurogenesis. The adult stress used consisted of a 20 minute long footshock session on 7 consecutive days. During each session, 5 uncontrollable and inescapable footshocks were applied (intensity: 0.8 mA; duration: 8 sec).

In this experiment, a 2 x 2 factorial design was used, with the factors control vs. maternal separation and control vs. adult stress, resulting in the 4 experimental groups: control (C), control-stress (CS), maternal separation (MS) and maternal separation-stress (MSS). Two different batches of 32 animals were used to study the effects of maternal separation and adult stress on explorative and anxiety-like behavior (first batch), cognition and social behavior (second batch). Data presented in the current paper was part of a larger experiment, in which other groups were used for a pharmacological study (unpublished data). Body weight and food intake were recorded throughout the experiment. Furthermore, adrenals and thymus were collected and weighed following termination on the last day of the experiment.

2.3.1 Behavioral tests

Open field The open field test is widely used to measure explorative behavior and anxiety in response to a novel environment (Hall, 1936). On day 2 of the 1-week adult stress period, the rats were placed in a large circular arena (120 cm diameter) for a period of 5 minutes. The open field arena was divided in two imaginary concentric circles with diameters of 40 cm and 120 cm, respectively: a central zone and an outer zone. Locomotion of the animals

was recorded and analyzed with a computerized imaging analysis system (Ethovision, Noldus Information Technology, Wageningen, the Netherlands). The following parameters were determined: latency entrance inner zone, frequency visits inner zone, time spent inner zone, total distance traveled. The open field arena was thoroughly cleaned between animals.

Elevated Plus Maze test The next day, the rats were subjected to an elevated plus maze, a commonly used anxiety test (Pellow et al., 1985). The plus maze consisted of two open arms and two closed arms arranged perpendicularly and elevated 55 cm above the floor. Each arm was 45 cm long and 10 cm wide. The closed arms had 50 cm high walls. At the start of the test the animals were placed on the centre of the maze facing an open arm. The test lasted 5 min and the behavior and locomotor activity of the animals was recorded on video for later analysis (Eline software; University of Groningen, The Netherlands). The following parameters were determined: (1) time spent in the open and closed arms and in the centre; and (2) number of visits to the closed and open arms. The plus maze was thoroughly cleaned between animals.

Novel object recognition In the second batch of animals, rats were subjected to a novel object recognition test (Ennaceur and Delacour, 1988). Half of the animals were tested on day 3 of the adult stress period, the other half on day 4. The test consisted of 3 consecutive trials of 5 min each, with an intertrial interval of 5 minutes, during which the animals were placed back in their home cage. During the first trial the animals were habituated to the novel environment, an empty plastic box measuring 50x50x35 cm. In the second trial, the rats were presented with two objects of identical size, shape and color (blue plastic cube). In the last trial, one familiar object and a new object, differing in shape, size and color (yellow plastic pyramid) were placed in the box, after which the animals were again allowed to explore the setting for a period of 5 minutes. The bedding in the box was cleaned between animals. During the test the behavior of the animals was recorded on video for later analysis (Eline software; University of Groningen, The Netherlands). The following parameters were determined: percentage of time spent exploring the novel and familiar object, the discrimination index (time spent exploring novel - familiar object) and the discrimination ratio ((time spent exploring novel - familiar)/total exploration time). Exploration of the object was defined as the time spent sniffing or touching the object.

Social interaction test For the social interaction test (day 5 or 6 of the stress period; Thor and Holloway, 1982) a rectangular wooden box measuring 35 x 20 x 15.5 cm was used (figure 4A). The box was divided in three areas: a rectangular (33 x 6.5 cm) area, which is referred to as neutral zone, and 2 square social interaction zones (16 x 14.5 cm) which were separated by a wall (14.5 x 15.5 cm). For the social interaction unfamiliar Wistar rats were placed into a small wire mesh cage (19 x 15 x 14 cm) in the social interaction zones. The social interaction test consisted of 3 trials of 5 min each, with an intertrial interval of 5 minutes, during which the animals were placed back in their home cage. At the beginning of every trial the experimental animal was placed in the neutral zone. In the first trial the animals were habituated to the novel environment. During this trial only the two empty wire mesh cages in the social interaction zones were present. In the second trial, the experimental rats were presented with a conspecific which was randomly assigned to one of the two interaction zones. In the last trial, a second novel animal was introduced in the

empty interaction zone. The social interaction box was cleaned between animals. During the test the behavior of the animals was recorded and analyzed with a computerized imaging analysis system (Ethovision, Noldus Information Technology, Wageningen, the Netherlands) and recordings were also used for additional analysis (Eline software; University of Groningen, The Netherlands). The following parameters were determined: percentage of time spent on social interaction, percentage of time spent on social interaction with new vs. familiar animal and the frequency and duration of social interaction with familiar and new animal.

2.3.2 Brain collection and immunohistochemistry

On the last day of the experiment, animals were sacrificed 1h after the end of the footshock session. Animals were sacrificed by transcardial perfusion with saline, followed by 4% paraformaldehyde. Brains were removed from the skull, postfixed overnight and subsequently cryoprotected in a 30% sucrose solution. After freezing the brains with liquid nitrogen, 12 series of 30 μm thick sections spanning the whole hippocampus were cut on a cryostat microtome. Free floating sections were stored in 0.01 M PBS containing 0.1% sodium azide until further processing.

Brain sections were analyzed to establish the effects of maternal separation and adult stress on different aspects of adult hippocampal neurogenesis. Cell proliferation was assessed by means of immunohistochemical staining for Ki-67, a nuclear protein that is expressed during all phases of the cell cycle, except G0 (Scholzen and Gerdes, 2000). Differentiation of new cells was examined by staining and analysis of the expression of doublecortin (DCX), a microtubule-associated protein which is found in immature neurons (Couillard-Despres et al., 2005; Rao & Shetty, 2004). Survival of new cells was assessed by staining for the exogenous thymidine analogue 5-bromodeoxyuridine (BrdU), which is incorporated into the DNA during the S phase of the cell cycle (Kee et al., 2002). To that purpose all animals received a single intraperitoneal injection with 100 mg/kg BrdU (50 mg/ml in saline, pH 7.0; Sigma, St. Louis, MO, USA) 2 weeks prior to the start of the adult footshock stress exposure.

For the Ki-67 and DCX staining, free-floating brain sections were treated with 0.3% H2O2 for 30 min, followed by incubation with 3% normal serum and 0.1% TritonX-100. Next, sections were exposed for 72 h to monoclonal mouse-anti-Ki-67 (1:200, Monosan, Uden, The Netherlands) or to polyclonal goat-anti-DCX (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA). After thorough rinsing, brain slices were treated with 3% normal serum and 0.1% TritonX-100, followed by 2 h incubation with biotinylated goat-anti-mouse for the Ki-67 staining or biotinylated rabbit-anti-goat for the DCX staining (1:400 for both antibodies, both from Jackson ImmunoResearch, Suffolk, United Kingdom). Subsequently, the avidine-biotin complex (ABC Elite kit, Vector Laboratories, Burlingame, CA) was added for 2 h, after which the staining was visualized with 0.2 mg/ml diaminobenzidine and 0.0033% H_2O_2.

The brain sections which were stained against BrdU were incubated consecutively for 30 minutes in 0.6 % H_2O_2 and for 2 h at 65 °C in saline sodium citrate (2x SCC)

containing 50% formamide. After successive rinses with 2x SCC, 2 M HCl (37 °C for 30 min), and 0.1 M borate buffer (pH 8.5), sections were exposed to the rat-anti-BrdU primary antibody (1:800, Oxford Biotechnology, Oxfordshire, UK) overnight at 4 °C. As secondary antibody, biotinylated donkey-anti-rat antibody was used (1:400, Jackson). Then, sections were incubated in streptavidin–horseradish peroxidase (1:200, Zymed) for 2 h at room temperature, rinsed, and reacted with DAB and H_2O_2. After the staining, the sections were mounted onto glass slides for microscopic analysis.

Ki-67 and BrdU positive labeled cells were counted in the subgranular zone of the hippocampus throughout the whole hippocampus at 40-times magnification. The number of positive cells was corrected for the length of the dentate gyrus. Results are shown as number of cells per mm. The optical density of DCX-positive cells and dendrites in the granule cell layer and the inner and middle molecular layer was measured throughout the entire anteroposterior extent of the hippocampus. A vast majority of the dendrites of the DCX positive cells extend into the middle molecular layer (Rao & Shetty, 2004). Optical density measurements were performed with a computerized system (Leica Qwin, Rijswijk, The Netherlands). An average value per section was calculated for each animal.

2.4 Statistical analysis

The ACTH and corticosterone levels and responses to different stressors and the body weight and food intake data were subjected to analysis of variance (ANOVA) with repeated measures, and when appropriate, a post hoc Tukey test was applied. For the statistical analysis of the adrenal and thymus weight, behavioral tests and the immunohistochemical data, two-way ANOVA and post hoc Tukey tests were used. In both cognitive tests some animals showed hardly any interest or no interest at all in the objects or the conspecific. Therefore data was tested for outliers by means of the Grubbs test. This test is based on the difference of the average and the most extreme data point considering the standard deviation (Grubbs, 1969). This resulted in the exclusion of one C and one CS animal in the social behavior test and one C and one MSS animal in the novel object recognition test. Furthermore, in the HPA-axis experiment one control animal died and therefore the data of this particular rat was excluded. Results are depicted as means with standard errors (SEM). Statistical significance was set at $P<0.05$.

3. Results

3.1 Effects of maternal separation on adult HPA-axis regulation

Figure 1 shows the ACTH and CORT levels and response to the different stressors in MS and C animals. Baseline levels of both ACTH and CORT appeared to be slightly higher in MS animals compared to controls on some of the days. However, this only reached statistical significance for the basal CORT level on the day of novelty exposure ($F_{1,13}=6.895$; $p=0.021$; figure 1B). The ACTH and CORT release in response to some of

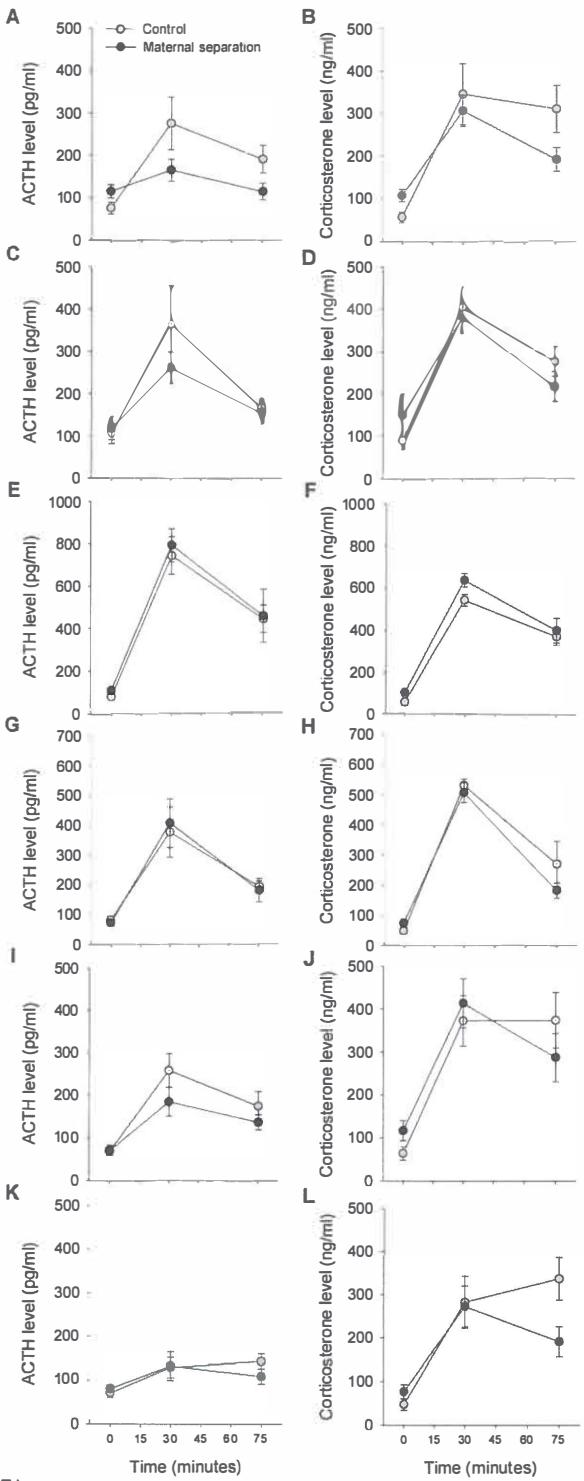

Figure 1 *Overview of the neuroendocrine response of MS and C animals during exposure to novelty (panel A-B), fox odor (panel C-D), footshock stress (panel E-F), re-exposure stress (panel G-H), and restraint stress (1 day: panel I-J; 5 days: panel K-L). The duration of all the stressors was 30 minutes. The ACTH and corticosterone levels at baseline (0 minutes), after the stress exposure (30 minutes) and after 45 minutes of recovery (75 minutes) are shown. Although baseline levels of ACTH and CORT seem to be higher in MS animals compared to C in all days measured, this only reached significance in the basal CORT levels before novelty exposure (p=0.021; panel B). The stress response to the different stressors seems to be lower in MS rats than in C animals. The ACTH response after novelty stress was significantly lower in the MS group (p=0.045; panel A). The neuroendocrine response to the other stressors showed no significant difference between MS and C. Stress habituation occurred in all animals, irrespective of the group (panel I-L).*

the stressors, if anything, seemed to be lower in MS rats. This was only significant for the ACTH response to novelty exposure ($F_{2,26}$=3.496; p=0.045; figure 1A).

To assess possible differences between MS and C rats in habituation to repeated stress exposure, the animals were exposed to restraint stress on 5 consecutive days. Habituation was observed in both C and MS animals, as can be seen by the attenuated ACTH and CORT responses after 5 days (respectively figure 1K and L) compared to the first day of restraint stress (figure 1I and J). However, the neuroendocrine responses to repeated restraint stress were not significantly different between groups.

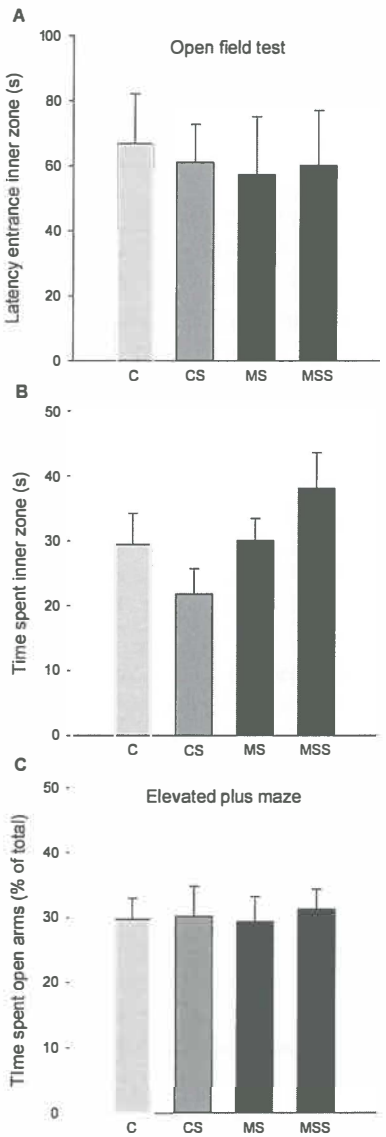

Figure 2 *Maternal separation did not change exploratory activity and anxiety-like behavior in response to a novel environment. There was no difference between MS and C in the latency of the entrance in the inner zone (A) or the time spent in the inner zone (B) of the open field, indicating no difference in exploratory activity and locomotion. The MS and C animals spent a similar amount of time on the open arms of the elevated plus maze, which implies that MS does not change anxiety-like behavior (C).*

Body weight was not influenced by maternal separation, but was significantly decreased in response to adult footshock stress ($F_{30,840}$=12.204; p<0.001; data not shown). Similarly, food intake was not different between maternally separated animals and controls, but was significantly decreased in the adult stress groups ($F_{9,252}$=2.721; p=0.005; data not shown). Adult footshock stress significantly increased relative adrenal weight independent of maternal separation ($F_{1,28}$=7.399; p=0.011; data not shown). Relative thymus weight was not changed by maternal separation or adult stress.

When adult animals were subjected to an open field test, there was no difference in exploratory activity between MS and C animals, and no effect of adult stress either. Maternally separated animals spent slightly more time in the inner zone of the open field than control rats but this difference did not reach statistical significance (2-way ANOVA effect of MS $F_{1,28}$=3.563; p=0.069; figure 2B). Also, in the elevated plus maze test, a more specific anxiety test, there was no significant effect of either maternal separation or adult stress (figure 2C).

Although activity and exploration in a novel environment, as measured in the open field test and the elevated plus maze test, were not affected by maternal separation or adult stress, it did influence the behavior of the animals in the novel object recognition task. During the object recognition test, the C animals spent significantly more time with the new object compared to the old one ($F_{1,12}$=6.931; p=0.022; figure 3), while in the maternal separation and the adult stress groups there was no clear preference for either of the objects. This might indicate an affected cognitive functioning in the maternally separated and stressed animals.

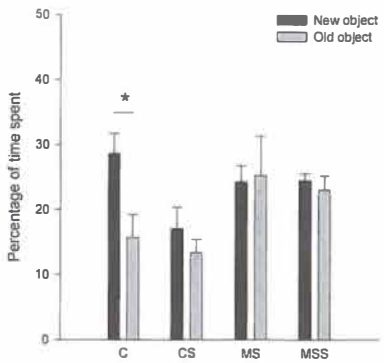

Figure 3 *The effect of maternal separation and adult stress on cognition. As expected, C animals showed a clear preference for the new instead of the old object in the novel object recognition test (p=0.022). In the other three groups there was no clear preference for the old or new object, indicating a cognitive impairment caused by maternal separation and adult stress.*

The results of the social recognition test were complex. In the first trial, animals of the different treatment groups did not differ in their exploration of the test environment (data not shown). In the second trial of the test, when one of the interaction zones contained a conspecific, the social interaction per se did not appear to be affected by MS or adult stress (figure 4B). All animals spent significantly more time in the zone with the unfamiliar novel animal than in the empty zone (C: $F_{1,12}$=53.729; p<0.001; CS:

$F_{1,12}$=41.079; p<0.001; MS: $F_{1,14}$=153.902; p<0.001; MSS: $F_{1,14}$=56.039; p<0.001). However, during the last trial, when the second interaction zone contained a novel conspecific, only animals previously exposed to adult footshock stress displayed a preference for this new compared to the old conspecific (CS: $F_{1,12}$=12.709; p=0.004; MSS: $F_{1,14}$=4.651; p=0.049; figure 4C). Both the non-stressed control animals and the non-stressed MS animals divided their time equally among the old and novel conspecific. The absence of a preference in the control and maternal separation group might suggest an undiminished interest in both animals rather than a cognitive impairment in these groups.

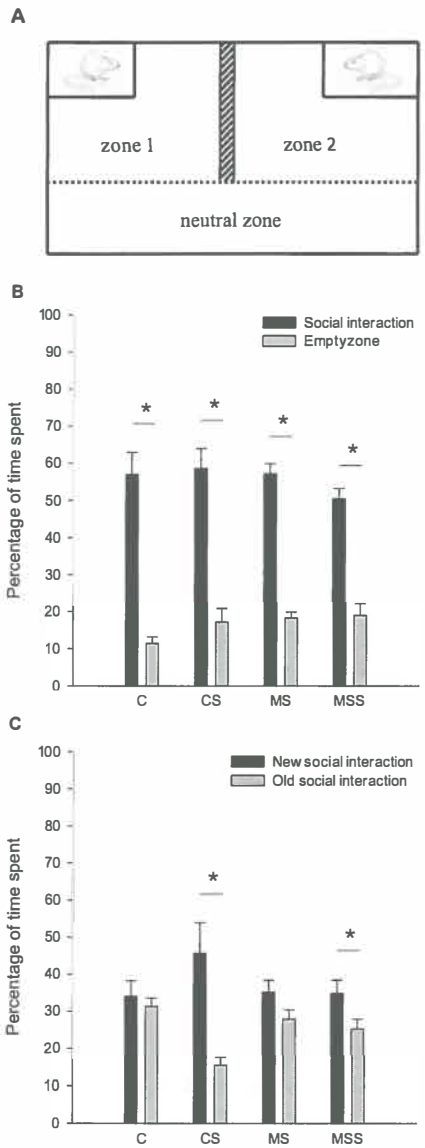

Figure 4 *The effect of maternal separation and adult stress on social interaction and social recognition. During the social interaction test the animals were placed in a box consisting of 3 different areas: a rectangular area, which is referred to as neutral zone, and two square social interaction zones which were separated by a wall (panel A). For the social interaction unrelated, unfamiliar conspecifics were placed into a small wire mesh cage in the social interaction zones. All animals showed a clear preference for the social interaction instead of the empty zone (B), hence maternal separation and adult stress did not influence social interaction per se. During the next trial, C animals had an undiminished interest in the old social stimulus and showed no preference for either the old or new conspecific (C). Similarly, MS rats showed no difference in the time spent with the old or the new social interaction. Interestingly, both adult stress groups (CS and MSS) showed a clear preference for the new social stimulus compared to the old one (CS: p=0.004; MSS: p=0.049).*

The effect of maternal separation and adult stress on the proliferation, neuronal differentiation and survival of newborn cells in the dorsal and ventral hippocampus was studied by staining for respectively Ki-67, DCX and BrdU (figure 5). The proliferation of new cells in the dorsal hippocampus was neither changed by maternal separation nor by adult stress (figure 5E). However, in the ventral hippocampus a clear decrease in the number of Ki-67 positive cells was seen in the adult stress groups compared to the non-stressed groups (2-way ANOVA $F_{1,27}=4.325$; $p=0.047$; figure 5F). Furthermore, there was a significant treatment interaction effect for maternal separation and adult stress (2-way ANOVA treatment interaction; $F_{1,27}=5.055$; $p=0.033$). Subsequent post-hoc analysis showed a significant difference between C and MS ($F_{1,13}=6.175$; $p=0.027$) and also adult stress was found to significantly decrease cell proliferation (C vs. CS: $F_{1,14}=10.177$; $p=0.007$). There was no difference between the number of Ki-67 positive cells between the MS and the MSS group. Adult stress did not aggravate the decrease in cell proliferation after maternal separation.

Unlike the number of proliferating Ki-67 positive cells, the number of surviving BrdU-labeled cells and the expression of DCX were not affected by MS or adult stress neither in the dorsal nor in the ventral hippocampus (figure 5G-J).

4. Discussion

In the present study, maternal separation did not substantially alter adult HPA axis reactivity as measured in a wide variety of conditions, nor did it change explorative activity and anxiety in the open field and elevated plus maze test or influence social recognition. Apparently, in contrast to general belief, the mother-infant interaction in rats can be rather strongly manipulated during a critical developmental phase without having major long-term consequences for stress sensitivity and emotionality.

While the prevailing idea still seems to be that MS causes long-lasting changes in neuroendocrine regulation and increased stress sensitivity after maternal separation (e.g. Huot et al., 2002; Plotsky et al., 2005), several other studies did not confirm this (e.g. Daniels et al., 2004; Slotten et al., 2006). In the present study, MS did not increase HPA axis activity and reactivity nor did it influence stress habituation, as measured by the response to repeated restraint. Importantly, we demonstrate that the variation between studies is not likely related to the nature of the stressor applied in adulthood. The lack of effect of MS in our study was consistent across a wide variety of adult stressors, from mild to severe, emotional as well as physical.

Also, in our study MS did not affect explorative activity and anxiety-like behavior in adulthood, neither did it increase the sensitivity to develop anxiety-like behavior under conditions of chronic stress exposure. Repeated exposure to footshock stress during adulthood also did not lead to increased anxiety in MS animals as compared to controls. While it has been reported that MS may lead to increased anxiety-like behavior (Aisa et al., 2007; Huot et al., 2002; Wigger et al., 1999), or even decrease anxiety (Maniam et al., 2010), our finding of unchanged anxiety-like behavior is in accordance with some previous studies (Aisa et al., 2007; Shalev et al., 2002; Slotten et al., 2006).

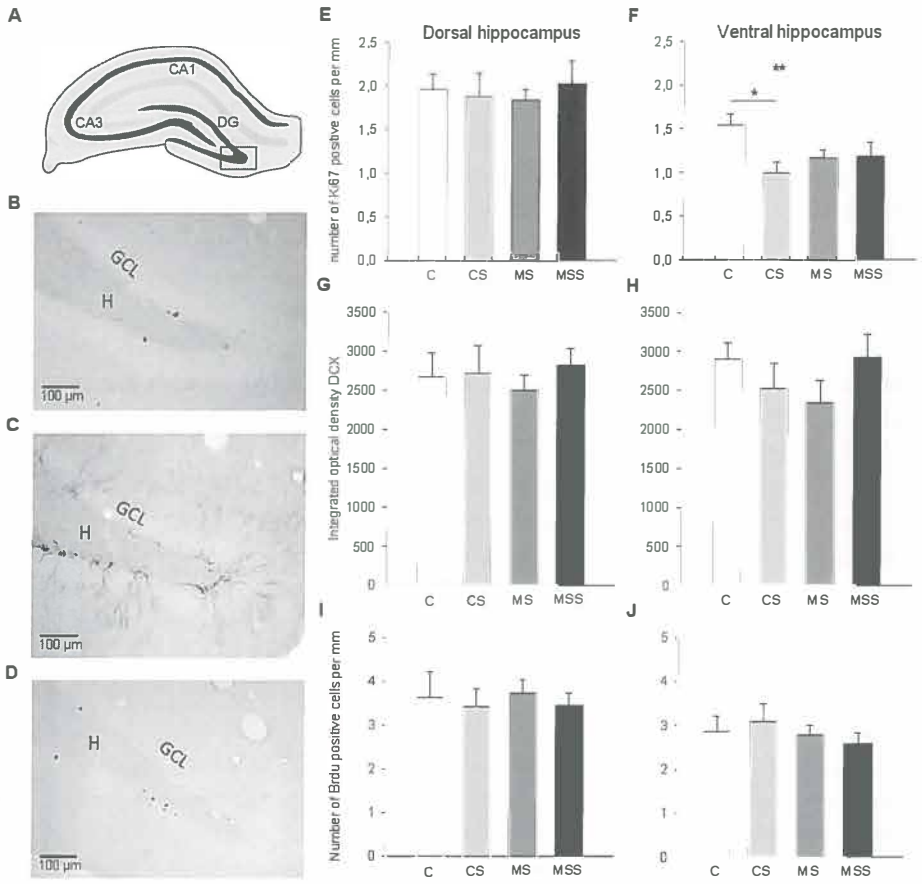

Figure 5 *The effect of maternal separation and adult stress on the proliferation (Ki-67), neuronal differentiation (DCX) and survival (BrdU) of newborn cells in the hippocampus. In panels B-D representative images (selected area shown in A) of respectively the Ki-67, DCX and BrdU staining are shown. Maternal separation and adult stress did not change the number of Ki-67 positive cells in the dorsal hippocampus (E). In the ventral hippocampus both CS and MS showed a decrease in cell proliferation compared to C (CS: p=0.007; MS: p=0.027; panel F). Unlike cell proliferation, cell differentiation was not found to be affected by maternal separation or adult stress, since there was no clear difference in the optical density (IOD) of DCX between the 4 groups (G-H). Also cell survival remained unchanged by maternal separation and adult stress (I-J).*

To examine cognitive function, the experimental animals were subjected to a novel object recognition test and a social recognition task. The novel object recognition test showed that short-term memory and object recognition was impaired as a consequence of both maternal separation and adult footshock stress. This is in accordance with a previous study showing an impairment of water maze learning and object recognition after maternal separation (Aisa et al., 2007).

The results of the social interaction test, a more complex cognitive task, were somewhat unexpected. In the first part of the test, animals in all treatment groups displayed

a strong interest in the interaction zone that contained a conspecific, thereby indicating that MS or adult stress did not increase fear or anxiety in such a context. Then, when 5 min after the first trial the second zone also contained a test rat, only the animals previously exposed to stress displayed a preference for this new animal. Both the non-stressed control animals and the non-stressed MS animals divided their time equally among the old and the novel conspecific. The object recognition task clearly shows that non-stressed control animals in a similar setup remember and distinguish old and new objects. Therefore, while we had anticipated that control animals would display a preference for the novel conspecific, the lack of such a preference might reflect the complexity of the social recognition task vs. the object recognition task. Since it is suggested that stress exposure prior to a learning task improves learning performance (Stamatakis et al., 2008), this might explain the (better) social recognition in the adult stress groups compared to the non-stressed animals. Yet, it then remains puzzling as to why this improved performance in stressed animals was absent in the novel object recognition test.

Granule cell neurogenesis in the dentate gyrus starts during late embryogenesis and continues during the early postnatal period (Altman & Bayer, 1990b). MS was carried out in the same period and might therefore interfere with the normal development of the hippocampus during this vulnerable time. Studies on the effects of maternal separation for 3h daily during the first few weeks of life on cell proliferation and survival during adulthood are rather scarce. In the current study, both maternal separation and adult stress significantly reduced cell proliferation in the ventral hippocampus, but did not influence the proliferation of new cells in the dorsal hippocampus. Footshock stress did not add to the reduction in cell proliferation which was already present in the ventral hippocampus of MS rats. The survival of new cells, as indicated by the number of cells labeled with BrdU, and the differentiation of new cells into neurons, as assessed by the expression of DCX, were not affected by MS and/or adult footshock stress. Previous studies have reported conflicting results on the effect of a 3h daily maternal separation paradigm on cell proliferation. In agreement with our finding, two other reports suggested a decrease in cell proliferation in maternally separated animals (Aisa et al., 2009; Mirescu et al., 2004), while another study observed no change in cell proliferation compared to controls (Nair et al., 2007). These studies did not distinguish between the dorsal and ventral hippocampus. It has been suggested that the dorsal and ventral hippocampus are involved in different functions. The dorsal hippocampus is mainly associated with spatial learning and memory, while the ventral portion of the hippocampus seems to be more involved in emotional and neuroendocrine functioning (Moser & Moser, 1998). However, the functional implication of the observed decrease in cell proliferation in the ventral hippocampus as a result of both maternal separation and adult stress is unknown.

The absence of MS effects on cell survival in our experiment is in agreement with a previous study, showing similar survival rates in control and maternally separated animals 3 weeks after BrdU injection (Mirescu et al., 2004). However, in the same paper a significant decrease in the number of BrdU positive cells was shown in the MS group one week after injection. Altogether, these data indicate that the effects of MS on the new cell population might be only transient.

Maternal separation and adult stress decreased cell proliferation, though only in the ventral hippocampus, but did not influence the number of surviving cells. Although the pool of new born cells appears to be smaller in MS animals, these cells might be more robust, and therefore higher numbers may survive and mature.

The highly variable outcome on different measures after maternal separation may depend on a variety of other factors. One possible factor is the diversity in methodological protocols for MS itself. For example, while in most studies the term maternal separation refers to mother-litter separation, in some studies the manipulation in fact also consists of isolation from littermates, next to separation from the mother (Biagini et al., 1998; Ladd et al., 1996; McIntosh et al., 1999). This latter manipulation has been suggested to constitute a more severe early life stressor and therefore lead to different consequences than maternal separation (Pryce & Feldon, 2003). Other contributors to the observed variety in literature might be the duration and frequency of the separation and the exact postnatal phase in which the separation takes place (for review see Lehmann & Feldon, 2000). Also, the strain of rats that is used for the MS studies and the genetic background on which the early life stress takes place may influence the outcome (Ellenbroek & Cools, 2000).

Another factor that may affect the outcome of MS, is the control group used in a study. Control groups most often used are either a "standard" or normal animal facility rearing (AFR) control group, as used in the current study, or early-handling (EH). During early handling, dam and litter are briefly separated for a period of up to 15 min, which leads, relative consistently, to HPA-axis hyporesponsiveness, a decreased level of anxiety and enhanced learning performance compared to AFR controls (for review see Lehmann & Feldon, 2000). Since EH and MS show opposite effects, the use of EH as control group may lead to larger effects which would have remained unnoticed when using AFR controls. As such, the choice of the control group may critically determine whether or not an effect of MS is found.

Even when only considering MS paradigms which us the same 3h daily MS protocol, the results are inconsistent. The variety observed in the literature discussed in the current paper, which only mentions the studies which applied the same protocol and control group as used in our lab, might be explained by the fact that a certain percentage of animals might be resistant to the effect of maternal separation. Oitzl and colleagues suggest that 40% of the animals are nonresponsive, while Sánchez et al. even suggest that up to 80% of the animals may be resistant to the negative impact of maternal separation (Oitzl et al., 2000; Sánchez et al., 2001). Although the maternal separation protocol described in these two papers is different from the current one, the dam and litter were separated for 24 hours on postnatal day 3, this observed resilience might be more general for all maternal separation models. This resistance to the effects of MS might be partly caused by (within litter) variation in maternal care (Champagne et al., 2003). The consequences of MS may depend on the balance between prolonged absence of the mother and subsequent maternal care that is received to compensate for it (Macri et al., 2004). In our study we did not examine the maternal care and cannot comment on the variation herein.

In conclusion, the present study shows that daily 3h MS during the first postnatal weeks in Wistar rats affects some aspects of adult neuroplasticity (hippocampal cell

proliferation) and cognitive function (object recognition). However, it did not lead to a hyperresponsive HPA-axis and increased anxiety as reported in some other studies. Wistar rats may be rather resistant to disturbances during early life or the effects of this maternal separation paradigm might be transient and dissipate during adulthood. Clearly, interference with the mother-infant relationship during early development does not necessarily lead to persistent changes in adult stress sensitivity and emotionality in the offspring.

Acknowledgments

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4.

Stress induces minor changes in sleep-wake behavior in both male and female Wistar rats

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Aloe Blacc - I need a dollar

Abstract

Epidemiological studies revealed a higher frequency of sleep complaints in females than in males, which may in part depend on sex differences in stress sensitivity and reactivity. In the present study we examined male-female differences in stress-induced changes in sleep architecture in rats after exposure to an acute physical stressor (footshock) and an emotional stressor (re-exposure to the shock box). In a parallel experiment the hypothalamic-pituitary-adrenal (HPA) axis response to footshock and re-exposure stress was assessed by measuring plasma levels of adrenocorticotrophin (ACTH) and corticosterone (CORT). To prevent fluctuating estradiol levels during the experiment, female rats were ovariectomized and received subcutaneous estradiol-releasing pellets to achieve constant, physiological estrogen levels. Under baseline conditions, the sleep-wake pattern showed no differences between the sexes. Stress exposure caused small changes in sleep-wake behavior in both males and females. In male rats, footshock stress suppressed non rapid-eye-movement (NREM) sleep duration in the first two hours. This was followed by a 1-2h period of increased NREM sleep EEG slow wave activity (SWA), suggesting increased sleep intensity to compensate for the sleep that was lost. Also, NREM sleep time was increased for a few hours in the middle of the subsequent dark phase. In female rats, NREM sleep time was only suppressed during the actual exposure to footshock stress, which was followed by a compensatory increase in EEG SWA immediately thereafter. The amount of rapid-eye movement (REM) sleep was suppressed for an additional hour after stress exposure. Re-exposure to the shock box the next day resulted in minimal changes in sleep architecture in both sexes. The inhibition of sleep during the actual stress sessions was followed by a small increase in EEG SWA thereafter. Female rats showed higher basal and stress-induced adrenal activity than males. In conclusion, despite a pronounced neuroendocrine stress response in both sexes, the stress-induced changes in sleep regulation appear only minimal. Furthermore, although female rats exhibited a higher adrenal activity, the alterations in sleep architecture after stress exposure, if anything, were more prominent in males.

Introduction

Epidemiological studies showed a clear sexually biased prevalence of sleep complaints and pathologies (for review, see Krishnan & Collop, 2006). Although men and women show similar baseline sleep patterns, females more frequently report sleep complaints (Bixler et al., 1984; Dijk et al., 1989; Goel et al., 2005; Roehrs et al., 2006). It has been suggested that primarily subtle male-female differences in sleep architecture increase in magnitude under stressful environmental challenges (Manber & Armitage, 1999). Sex differences in stress sensitivity might be related to the clear female predominance of sleep-related complaints.

The use of animal models provides the opportunity to study how stress influences sleep, and whether these stress effects are different for males and females, under controlled experimental settings. So far, few studies in rodents were specifically devoted to investigating sex differences in stress-induced alterations in sleep architecture. Similar to humans, the male-female differences in baseline sleep patterns in rodents are subtle. Males exhibit more non rapid-eye movement (NREM) sleep and/or more rapid-eye-movement (REM) sleep than females (Yamaoka, 1980; Fang & Fishbein, 1996; Koehl et al., 2006; Paul et al., 2006; Tiba et al., 2008). Other studies reported higher EEG slow wave activity (SWA), an indicator of sleep intensity, in females (Ehlers et al., 1993) and higher levels of NREM sleep time specifically in diestrus females (Andersen et al., 2008) when compared to male rats. The sex differences in sleep architecture observed in these papers may in part be related to the influence of sex steroids, since gonadectomy in male and female mice eliminated the differences in sleep-wake behavior (Paul et al., 2006). Several studies described subtle estrous cycle-related differences in sleep patterns, showing a reduction in REM, and frequently NREM sleep during the proestrus phase, the time of high estrogen secretion (Nequin et al., 1979; Zhang et al., 1995; Fang & Fishbein, 1996; Schwierin et al., 1998; Hadjimarkou et al., 2008). However, not all studies reported such changes in sleep patterns across the estrous cycle (Andersen et al., 2008). Moreover, a study by Koehl and colleagues demonstrated that the occurrence of changes in sleep across the estrous cycle in female mice is strain-dependent (Koehl et al., 2003).

A substantial number of studies has assessed the effects of stress on sleep in male rodents (e.g. Meerlo et al., 1997; Meerlo et al., 2001; Koehl et al., 2002; Tang et al., 2007). However, studies on sex differences in the stress-induced alterations of sleep are rather scarce. The stress associated with acute exposure to a low temperature (for 1h at 4°C) suppressed subsequent REM sleep duration in male and female rats, while NREM sleep duration was increased in both sexes. Subsequently, nocturnal sleep time and REM sleep time in both male and female rats was increased, while a NREM sleep rebound was induced only in females (Tiba et al., 2008). In two other studies, the exposure of male and female mice to restraint stress at the beginning of the light phase was followed by an initial reduction in NREM and/or REM sleep time and subsequently an increase in REM sleep during the dark phase in both sexes (Koehl et al., 2006; Paul et al., 2009b), although a stronger REM rebound was observed in males (Koehl et al., 2006). The subtle variation in these stress-induced changes in sleep might be explained by the nature of the stressor applied.

The stress-induced sex differences in sleep architecture might be partly related to male-female differences in HPA axis regulation. Several studies reported sex differences in basal and stress-induced neuroendocrine levels, with higher levels in females than in male rats, independent of the type of stressor (Kitay, 1961; Critchlow et al., 1963; Atkinson & Waddell, 1997; Shors et al., 1999; Hulshof et al., submitted).

The primary aim of the present study was to obtain a clearer picture on the male-female differences in stress-induced changes in sleep architecture in rats. Since changes in estradiol during the estrous cycle might influence both HPA-axis regulation and sleep, females were ovariectomized and equipped with pellets warranting constant physiological levels of estradiol throughout the experiment. To assess if sex differences in stress-induced changes in sleep-wake behavior depend on the nature of the stressor, we subjected males and females to a paradigm normally used for fear conditioning, consisting of a footshock exposure (physical stressor) and a re-exposure to the context on the subsequent day (purely emotional stressor).

Methods

Animals and experimental outline

The study was performed in adult age-matched male and female Wistar rats (Harlan, Horst, The Netherlands). All animals were individually housed under a 12h/12h light-dark cycle with temperature maintained at 21±1°C. Food and water was available ad libitum. The experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Besides studying the stress-induced changes in sleep architecture, sex differences in HPA-axis responses to stress were assessed in a separate experiment. All animals were subjected to stress during the first hours of the light phase, when basal activity of the HPA-axis is at its lowest (e.g. De Boer & van der Gugten, 1987; Atkinson & Waddell, 1997; Windle et al., 1998). In both experiments, all male and female groups consisted of 8 animals.

Ovariectomy and estradiol replacement

Since changes in estradiol during the estrous cycle might influence both HPA-axis regulation and sleep, female rats were bilaterally ovariectomized (OVX) and equipped with tablet-shaped pellets (approx. 1 cm in diameter and 3-4 mm thick) to achieve constant physiological levels of estradiol throughout the experiment (β-estradiol 3-benzoate in cholesterol, both from Sigma, St Louis, MO, USA). After surgery and pellet implantation the animals were allowed to recover for at least 10 days.

At the end of both experiments blood samples were taken to measure estradiol levels (radioimmunoassay; MP Biomedicals, Orangeburg, NY, USA). The implantation of the pellets after OVX resulted in blood estradiol levels of 103.9 pg/ml ± 7.1 pg/ml in the HPA-axis experiment and 111.2 pg/ml ± 14.1 pg/ml in the sleep experiment. As

previously measured, the endogenous estradiol concentration during the different stages of the estrous cycle ranged between 67.3 ± 3.0 pg/ml and 126.6 ± 3.9 pg/ml (Hulshof et al., submitted). Therefore, the female rats in the present study obtained high physiological levels of estradiol, comparable to the levels observed during the proestrus phase of the estrous cycle.

Stress paradigm

To study sex differences in stress on HPA-axis activity and sleep, male and female rats were subjected to a fear conditioning paradigm. On the first day, the animals were subjected to footshock stress (a severe physical stressor), during which the rats were placed into the footshock box, immediately after lights on, and received 5 shocks, each of a duration of 3 seconds and an intensity of 0.8 mA. On the next day, the animals were re-exposed to the footshock box (a purely emotional stressor, based on conditioned fear), again at lights on, without administering footshocks.

Experiment 1: HPA-axis activity

In the first experiment the neuroendocrine response to footshock and re-exposure stress was assessed. Blood samples were taken at three time points: prior to the stress exposure (baseline), directly after a 30 minutes stress exposure (peak HPA axis response) and 45 minutes after the stressor (recovery of the stress response). Blood samples of approx. 0.5 ml were drawn from the tail and collected in prechilled cups containing EDTA as anticoagulant. Thereafter, samples were centrifuged at 4°C for 15 minutes at 2600 g, and the supernatant was stored at -80°C until further analysis. ACTH and CORT levels were determined by radioimmunoassays (MP Biomedicals, Orangeburg, NY, USA).

Experiment 2: Sleep-wake patterns

To measure sleep-wake architecture, rats were equipped with EEG and EMG electrodes (Plastics One, Roanoke, VA, USA) under deep surgical anesthesia (O_2/NO_2 and isoflurane). Stainless steel screws for EEG recordings were placed epidurally on contra-lateral positions (2 mm lateral to the midline, 1 mm anterior to bregma on the left side and 3 mm posterior to bregma on the right side). One additional screw (1 mm anterior from bregma on the right side) functioned as grounding. Stainless steel wires inserted in the nuchal muscle were used to record EMG activity. In the females, the OVX and the placement of the EEG and EMG electrodes were combined in one surgery. After surgery the animals were allowed to recover for at least 10 days before starting baseline sleep-wake recordings.

During the recovery period after EEG/EMG surgery, the animals were habituated to the recording procedures. For that purpose, rats were connected to the recording cables for 3 consecutive days before the start of the experiment, to assure that the cable would not disturb the animals' sleep and to prevent possible unwanted stress

caused by the handling of the animals. Before the fear conditioning paradigm basal 24h sleep-wake patterns were recorded in all animals. After the 1h lasting exposure to footshock or re-exposure stress, sleep patterns were recorded during the remaining 23h of the day.

The EEG and EMG signals were monitored using a recording unit (Vitaport 3, 2x8 channel universal recorder, TEMEC Instruments, Kerkrade, The Netherlands) connected to a pc running a data acquisition and storage program (Columbus V 1.09.05, TEMEC Instruments, Kerkrade, The Netherlands). The animals were connected to the recording unit via a commutator that allowed free movement throughout the home cage. EEG signals were amplified 5,000x with high and low pass filters at respectively 0.5 and 30 Hz. EMG signals were amplified 2,000x with high- and low pass filters at 0.5 and 20 Hz to prevent AC noise to affect the signals. Signals were stored at 128 Hz on a flash disk in the recording unit for further processing.

Sleep data was analyzed using Vitascore software (V 1.30, TEMEC Instruments, Kerkrade, The Netherlands). Ten second epochs were rated visually according to classical sleep-wake parameters for wakefulness (low amplitude EEG and high EMG activity), NREM sleep (high amplitude EEG and low EMG activity) and REM sleep (low amplitude EEG and low EMG activity; Meerlo & Turek, 2001). For the quantitative analysis of EEG, the signals were subjected to fast Fourier transformation in ten second bins. NREM sleep EEG power in the 0.5 - 5 Hz delta band was calculated as a measure of NREM sleep intensity (Borbély et al., 1981). To correct for inter-individual differences in EEG signal strength, the delta power values were normalized to the average NREM sleep EEG delta power of the baseline recording. The normalized EEG delta power is referred to in the text and figures as slow wave activity (SWA).

Statistical analysis

The neuroendocrine response to footshock and re-exposure stress was analyzed by subjecting the ACTH and CORT data to analysis of variance (ANOVA) with repeated measures. Baseline sleep architecture of males and females was compared by repeated measures ANOVA separately for the light and dark phases. Changes in sleep pattern caused by the stress exposures were analyzed by repeated measures ANOVA, again separately for the light and dark phase. When repeated measures testing showed significant effects, a post hoc Tukey test was applied. Total sleep and wake time and SWA per day and per 12h interval were analyzed by 2-way ANOVA. Sex differences in sleep architecture in response to both stressors were analyzed by one-way ANOVA. Results are depicted as means with standard errors (SEM). Statistical significance was set at $P<0.05$.

Results

HPA-axis activation

There was no significant difference in stress-induced ACTH release between males and females, neither in response to footshock stress nor upon the re-exposure to the shock box the next day. In contrast, repeated measures ANOVA revealed a significant sex difference, with higher levels in females, for the CORT response to both footshock stress (overall sex effect: $F_{1,13}=34.01$, $p<0.001$; sex x time interaction: $F_{2,26}=3.63$, $p=0.041$) and shock box re-exposure (overall sex effect: $F_{1,13}=13.01$, $p=0.003$). In particular, females had higher baseline CORT levels and higher peak response levels than males ($p<0.02$ for both; figure 1).

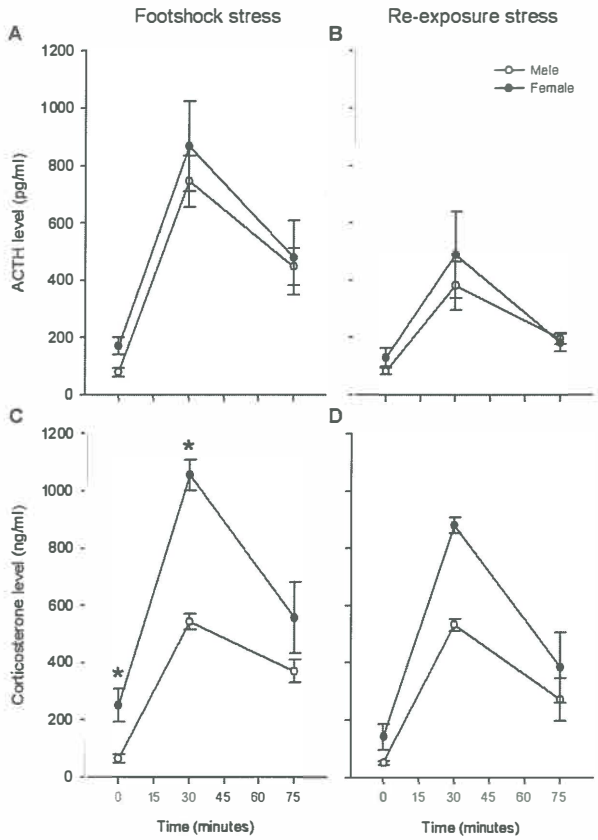

Figure 1 *The neuroendocrine response in males and females during the conditioned fear paradigm. The ACTH and corticosterone levels at baseline (0 minutes), after 30 minutes of stress exposure (30 minutes) and after 45 minutes of recovery (75 minutes) are shown. In response to footshock stress females showed significantly higher CORT levels compared to males. The ACTH levels were not significantly different between sexes during either footshock or re-exposure stress. *significant differences between male and female rats ($p<0.05$).*

Male rats

In one male rat the EEG/EMG signal was lost during sleep measurements after footshock stress. This animal therefore had to be excluded from the analysis. The exposure to footshock stress significantly affected the amount of wakefulness during both the remainder of the light phase (stress x time interaction: $F_{10,130}=2.19$ $p=0.022$) and the subsequent dark phase in male rats (stress x time interaction: $F_{11,143}=2.53$ $p=0.006$; figure 2D). Wakefulness was enhanced in the first hour following stress exposure as compared to baseline ($p<0.01$). During the dark phase, small differences in wakefulness in either direction were observed.

The increase in wakefulness directly following footshock stress appeared to occur at the expense of mainly NREM sleep. NREM sleep time was significantly altered after footshock stress during both the light phase (stress x time interaction $F_{10,130}=2.23$ $p=0.020$) and dark phase (stress x time interaction $F_{11,143}=2.29$ $p=0.013$; figure 2B). NREM sleep was significantly reduced during the first hour after stress exposure ($p<0.01$). A small, but significant NREM sleep rebound was observed during the dark phase ($p<0.04$ for both time points).

Slow wave activity, a measure for sleep pressure and sleep intensity, also showed significant changes during the light phase (stress x time interaction $F_{10,130}=5.40$ $p<0.001$) and dark phase (stress x time interaction $F_{11,143}=2.21$ $p=0.017$) after the exposure to footshock stress (figure 2A). After an initial significant decrease during the first hour after stress exposure ($p<0.01$), SWA subsequently increased until a significant peak at the third hour after the exposure ($p<0.01$). During the dark phase, changes in SWA were observed in either direction ($p<0.02$ for both).

In contrast to NREM sleep, REM sleep was only affected during the dark phase (stress x time interaction $F_{11,143}=2.99$ $p=0.001$; figure 2C). After an initial increase in the beginning of the dark phase ($p<0.02$), the time spent in REM subsequently decreased at the end of the dark phase ($p<0.01$ for both).

Overall, NREM and REM sleep duration and the total amount of wakefulness during the 23h period following footshock were not significantly different from the amount under baseline conditions.

Female rats

Females only showed minimal footshock stress-induced changes in sleep. In fact, the amount of wakefulness and NREM sleep duration following stress were not significantly altered (figure 2). SWA was significantly altered during the light phase (stress x time interaction $F_{10,140}=2.46$ $p=0.010$), showing an elevation above baseline levels lasting for several hours ($p<0.03$ for all; figure 2A). Similarly, footshock stress significantly influenced REM sleep during the light phase (stress x time interaction $F_{10,140}=2.30$ $p=0.016$; figure 2C). Females showed a reduction in time spent in REM directly following stress exposure ($p<0.05$).

Overall, the total amount of wakefulness, REM and NREM sleep duration and SWA during the 23h period following footshock were not significantly different from the amount under baseline conditions.

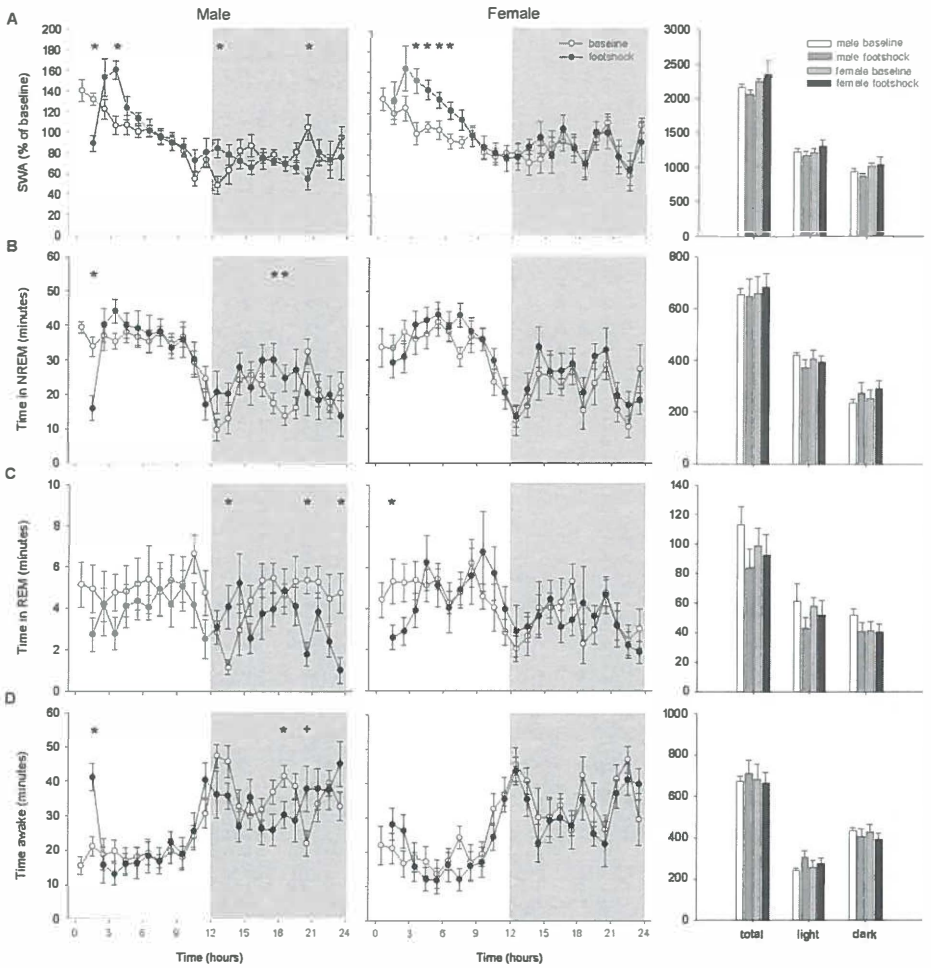

Figure 2 *The effects of footshock stress (1h) on the sleep architecture of male and female rats. The first two columns present the 24h sleep patterns for males and females. The gray area represents the 12h dark period. In the third column total SWA and total time spent in NREM, REM and awake are shown. *significant differences are indicated for p<0.05 (+ p=0.05).*

Re-exposure stress-induced changes in sleep architecture

Male rats

In males, the re-exposure to the footshock box significantly reduced the amount of wakefulness during both the light phase (overall stress effect $F_{1,14}$=5.51 p=0.034) and the dark phase (overall stress effect $F_{1,14}$=10.19 p=0.007; figure 3D). This stress-induced

decrease in wakefulness was mirrored by an increase in NREM sleep (light phase: overall stress effect $F_{1,14}=6.12$ $p=0.027$; dark phase: overall stress effect $F_{1,14}=12.57$ $p=0.003$; figure 3B).

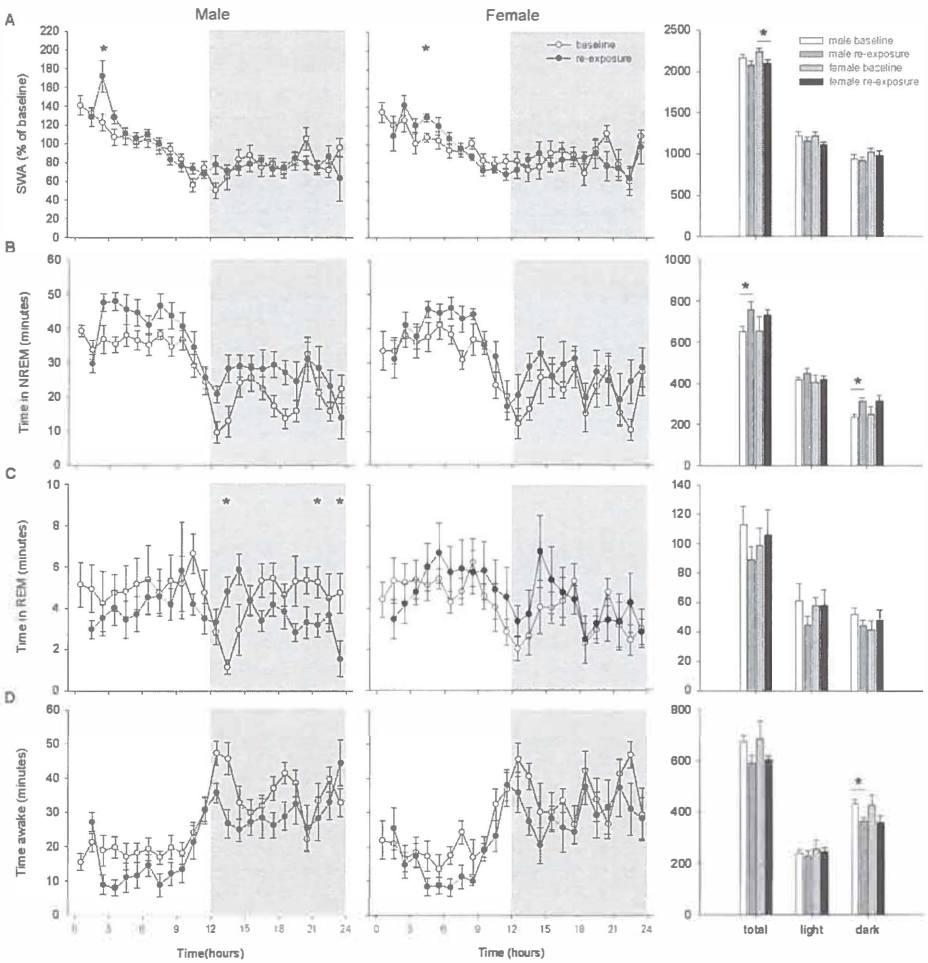

Figure 3 *The effects of re-exposure stress (1h) on the sleep architecture of male and female rats. The first two columns present the 24h sleep patterns for males and females. The gray area represents the 12h dark period. In the third column total SWA and total time spent in NREM, REM and awake are shown. * significant differences are indicated for $p<0.05$.*

SWA was significantly influenced in males during the light phase (stress x time interaction $F_{10,140}=3.73$ $p<0.001$), showing significantly higher levels during the second hour after stress exposure ($p<0.03$; figure 3A).

The time spent in REM sleep during the dark phase was also significantly changed by re-exposure stress (stress x time interaction $F_{11,154}=4.12$ $p<0.001$; figure 3C). While in

the beginning of the dark phase stressed male rats spent more time in REM sleep ($p<0.01$), at the end of the dark phase the males showed a decrease in REM sleep when compared to baseline ($p<0.05$ for both).

Overall, the total amount of wakefulness was only affected during the dark phase, where males spent significantly less time awake after re-exposure to the shock box when compared to baseline ($F_{1,14}=10.19$ $p=0.007$). Also the amount of wakefulness across the whole 23h recording period was slightly lower after re-exposure stress than under baseline conditions. This, however, did not reach statistical significance. The total NREM sleep duration showed a significant increase after stress exposure in males in the dark phase ($F_{1,14}=12.57$ $p=0.003$) and during the whole 23h recording period ($F_{1,14}=5.56$ $p=0.033$). The total amount of SWA and REM sleep duration were not significantly different between groups during the 23h recording period.

Female rats

In one of the females, the EEG/EMG signal was lost and this animal therefore had to be excluded from the analysis. Again female rats showed only minimal changes after stress exposure. The amount and patterns of wakefulness, NREM and REM sleep were not significantly influenced by re-exposure stress, neither in the light phase nor in the subsequent dark phase. However, SWA was significantly influenced during the light phase (stress x time interaction $F_{10,130}=1.92$ $p=0.048$), showing a small increase a few hours after stress exposure ($p<0.01$).

Overall, the amount of wakefulness and both NREM sleep and REM sleep duration were not significantly altered after re-exposure to the shock box. However, SWA over 23h was significantly reduced by stress exposure in females ($F_{1,13}=5.14$ $p=0.041$).

Sex differences in sleep architecture

Baseline sleep patterns, i.e. time spent in NREM, REM or awake and slow wave activity, did not show significant differences between sexes (figure 4). The deviation from baseline after footshock stress and re-exposure to the footshock box was calculated to analyze sex differences in stress-induced alterations in sleep-wake behavior. Only animals with both a baseline and stress measurement were included, therefore from the female footshock and re-exposure data one animal was excluded. Male-female differences appeared to be minimal after stress exposure. A significant difference between sexes was found in REM sleep during the light phase as a response to footshock stress ($F_{11,132}=1.92$ $p=0.042$; data not shown). Although not significant, female rats initially showed lower REM sleep levels than males, while at the end of the light phase time spent in REM sleep was higher in females. Total stress-induced changes from baseline were not significantly different between sexes for any of the parameters measured (figure 4).

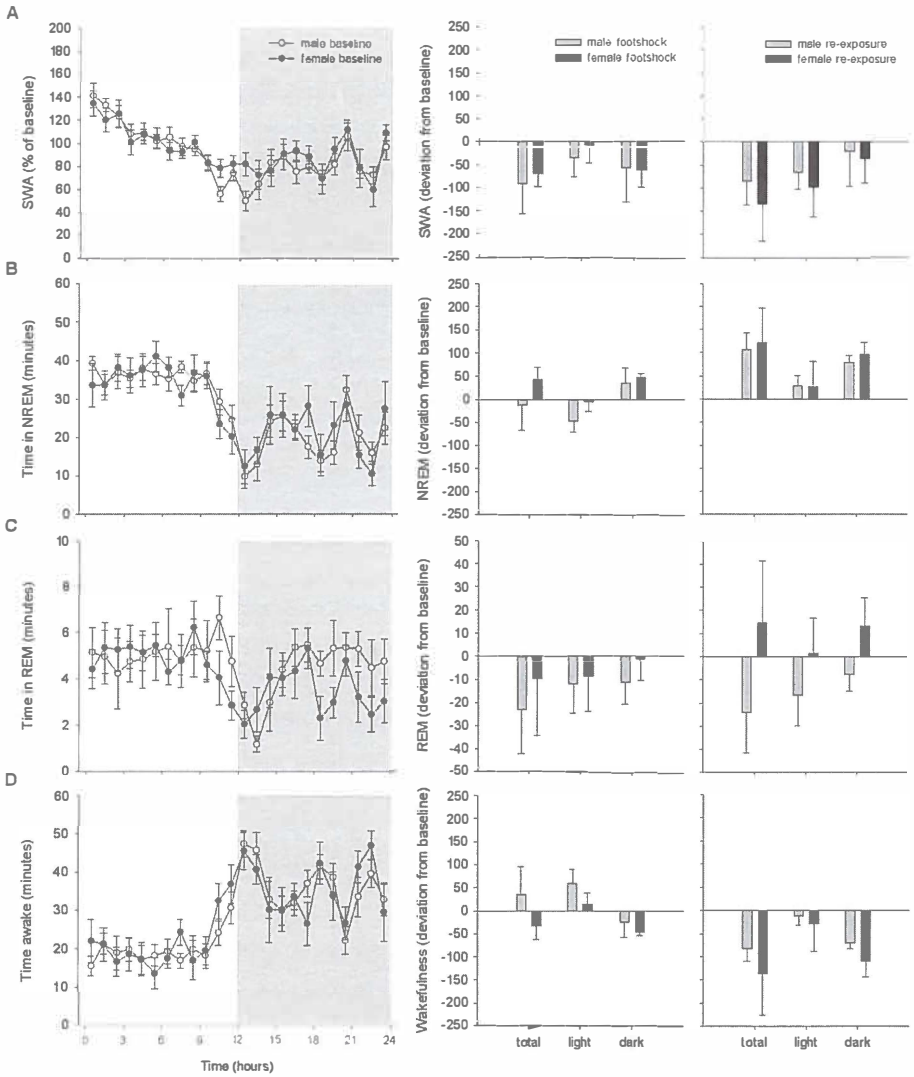

Figure 4 *Sex differences in baseline sleep and stress-induced changes in sleep architecture. The first column presents the baseline sleep in males and females (gray area represents 12h dark period). In the second and third column, the stress-induced changes relative to baseline are shown for both sexes for respectively footshock and re-exposure stress.*

Discussion

The primary aim of the present study was to investigate 1) sex differences in stress-induced changes in sleep architecture, and 2) the influence of the nature of the stress herein. The results show that male and female Wistar rats display little differences in sleep-wake architecture under baseline conditions and after a stressful challenge. Stress only had minor

effects on sleep-wake behavior in both males and females. In response to footshock stress, both sexes showed an initial decrease in sleep duration, which was subsequently followed by an increase in NREM sleep SWA. Only male rats showed a delayed NREM sleep rebound in response to footshock stress. The emotional stress associated with the re-exposure to the footshock box influenced sleep to an even smaller extend. Overall, despite a much higher adrenal activity and corticosterone responses in female rats compared to males, there were only modest sex differences in subsequent sleep. In fact, if anything, male rats appeared to be more sensitive to stress-induced changes in sleep than females.

Although several reports described the effects of stress on sleep in male rats, studies on the interaction between sex, stress and sleep are rather scarce. In the current study, stress only had minor effects on sleep-wake behavior in both sexes, despite the strong neuroendocrine response to stress. Males showed, concomitant with prolonged waking, an initial footshock stress-induced decrease in NREM sleep time and SWA, subsequently followed by an increase in SWA and a nocturnal NREM sleep rebound. In female rats, the wake-enhancing effect of footshock stress was basically limited to the actual footshock session, although female rats exhibited a small but significant reduction in REM sleep duration immediately following stress exposure. Also in females, footshock stress was followed by an elevation in SWA, but there was no significant effect on subsequent sleep-wake behavior. Re-exposure stress had even smaller effects on the sleep-wake patterns of male and female rats. Besides a small increase in SWA immediately following the re-exposure session in both sexes, re-exposure stress minimally affected subsequent sleep in male rats.

Similarly, a few previous studies reported minor stress-induced changes in sleep-wake behavior in male and female rodents. Tiba and colleagues showed an initial increase of NREM and decrease of REM sleep in rats in response to cold stress, followed by a nocturnal increase in total sleep time and REM sleep in both sexes (2008). In addition, female rats showed a nocturnal NREM sleep rebound. In response to restraint stress, male and female mice showed an initial reduction in REM and NREM sleep, subsequently followed by a nocturnal increase in NREM sleep in both sexes, while only males exhibited an increase in REM sleep during the dark phase (Koehl et al., 2006). Similarly, Paul and colleagues reported an initial restraint-stress induced reduction in REM sleep in both male and female mice, with a concomitant reduction in NREM sleep in females, which was followed by a nocturnal increase in REM sleep in both sexes (Paul et al., 2009b). Similar to the slightly stronger effects of stress on sleep in males in the present study, Koehl and colleagues also observed a stronger stress-induced effect on sleep in male mice (Koehl et al., 2006). Some of the discrepancy between the previously published studies and the current results might be related to the nature of the stressor the animals were subjected to or, in case of the females, the phase of the estrous cycle or the level of estradiol. The previously mentioned studies did either not distinguish between cycle phase, despite the possible influence of estradiol on sleep, or specifically used diestrus females.

In the present study, both male and female rats displayed a significant increase in SWA after footshock stress as well as re-exposure to the shock box. Considering that the rats were awake during the stress exposure, which took place during the beginning of the

light phase, the increased sleep intensity might just be related to sleep loss per se. However, a previous study by Meerlo and colleagues suggests that stress might induce an additional increase in SWA beyond what is seen after normal wakefulness (Meerlo et al., 1997). Indeed, stress-specific increases in SWA have been reported for e.g. social defeat stress and restraint stress (Meerlo et al. 1997; Tang et al., 2007). However, since in the current study a sleep deprivation control group was not included, we cannot distinguish between the effects of sleep loss and stress on SWA.

Also with regard to the subtle changes in sleep architecture following stress exposure, it remains uncertain whether these alterations are a normal response to sleep loss or a specific effect of stress. Although the absence of a sleep loss control does not allow us to draw conclusions, it is not excluded that some of the changes are stress specific. The subtle differences in sleep-wake behavior between male and female rats seem to support this. Such sex differences are more likely related to a difference in response to stress than a difference in response to sleep deprivation (Koehl et al., 2006). A number of earlier studies investigated the differences between the impact of sleep deprivation and stress exposure on subsequent sleep-wake behavior in male rodents. While sleep deprivation by gentle handling for 1 or 1.5h induced minor, short-lasting changes in sleep-wake patterns, the exposure to restraint and social stress affected sleep to a greater extent (Meerlo et al., 1997; Meerlo et al., 2001; Meerlo & Turek, 2001). Furthermore, another study reported that subjecting male rats to 3h of sleep deprivation by gentle handling at the beginning of the light phase did not affect SWA or REM sleep and had only minimal impact on NREM sleep (Tobler & Borbély, 1990).

If indeed some of the changes in sleep architecture are stress specific, they do not appear to be mediated by glucocorticoids. Despite the higher adrenal reactivity in female rats, the sleep-wake behavior was only minimally influenced by the stress exposure. In contrast, male rats showed small but clear stress-induced alterations in different sleep parameters. The mechanisms underlying the sex differences in the sleep-stress relation remain to be established. Although speculative, one of the factors that might play a role in the observed sex-differences is prolactin. Prolactin, which is elevated in response to stress (Meerlo et al., 2001), was found to increase REM, but not NREM, sleep duration (Roky et al., 1993). A few studies suggested that prolactin is essential for the stress-induced REM sleep rebound (Bodosi et al., 2000; Meerlo et al., 2001; Obál Jr et al., 2005). The stress-induced increase in pituitary prolactin mRNA appears to be different between sexes, with a more pronounced increase in male rats (Dave et al., 2000). Therefore, in the present study, stress might have increased prolactin mRNA in male rats and, if translated in protein, may explain the small REM sleep rebound observed in males.

In summary, in the present study male and female rats displayed little differences in basal sleep-wake architecture. Stress only had minor effects on sleep-wake patterns in both male and female rats, despite the substantial stress-induced neuroendocrine response. Whereas adrenal reactivity was significantly higher in female rats when compared to males, the sex differences in subsequent sleep were only modest. In fact, male rats appeared to be more sensitive to stress-induced changes in sleep than females. In this context, the reported

higher prevalence of sleep complaints in women cannot be explained by the sex difference in stress sensitivity of sleep observed in the current study.

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5.

Despite higher glucocorticoid levels and stress responses in female rats, both sexes exhibit similar stress-induced changes in hippocampal neurogenesis

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Behavioural Brain Research, submitted

The Black Keys - Tighten up

Abstract

Sex differences in stress reactivity may be one of the factors underlying the increased sensitivity for the development of psychopathologies in women. Particularly, an increased hypothalamic-pituitary-adrenal (HPA) axis reactivity in females may exacerbate stress-induced changes in neuronal plasticity and neurogenesis, which in turn may contribute to an increased sensitivity to psychopathology. The main aim of the present study was to examine male-female differences in stress-induced changes in different aspects of hippocampal neurogenesis, i.e. cell proliferation, differentiation and survival. Both sexes were exposed to a wide variety of stressors, where after differences in HPA-axis reactivity and neurogenesis were assessed. To study the role of oestradiol in potential sex differences, ovariectomized females received low or high physiological oestradiol level replacement pellets. The results show that females in general have a higher basal and stress-induced HPA-axis activity than males, with minimal differences between the two female groups. Cell proliferation in the dorsal hippocampus was significantly higher in high oestradiol females compared to low oestradiol females and males, while doublecortin (DCX) expression as a marker of cell differentiation was significantly higher in males compared to females, independent of oestradiol level. Stress exposure did not significantly influence cell proliferation or survival of new cells, but did reduce DCX expression. In conclusion, despite the male-female differences in HPA-axis activity, the effect of repeated stress exposure on hippocampal cell differentiation was not significantly different between sexes.

1. Introduction

Male-female differences in the basal and stress-induced levels of corticosterone (CORT) in rats were first reported in the 1960s (Kitay, 1961; Critchlow et al., 1963). These early studies showed higher basal CORT levels and a higher stress response after ether exposure in female rats than in males. Since then, several other studies which made a direct comparison between males and females found similar sex differences (e.g. Kant et al., 1983; Aloisi et al., 1994; Seale et al., 2004a). However, most of these studies did not distinguish between the different phases of the oestrous cycle even though others showed that CORT levels change with cycle stage in female rats, with peak levels during the proestrus phase, the time of high oestrogen secretion (Critchlow et al., 1963; Raps et al., 1971; Nequin et al., 1979). Furthermore, ovariectomy (OVX) was found to decrease basal CORT levels in females, while oestradiol administration reversed this OVX-induced decrease (Kitay, 1963). Considering the impact of oestradiol on neuroendocrine regulation, the phase of the oestrous cycle and the oestradiol level have to be taken into account when investigating sex differences in HPA-axis regulation. One study that compared males and intact cycling females showed higher basal CORT levels in females during the proestrus phase of the cycle (Raps et al., 1971). Another study observed higher levels of CORT in females than males during both proestrus and diestrus (Atkinson & Waddell, 1997). Moreover, the CORT response to footshock stress was higher in proestrus females than in males (Rivier, 1999). In contrast, others found that both basal levels of CORT and stress-induced increases in CORT where higher in females than males, independent of the phases of the female cycle (Shors et al., 1999).

Considering that females seem to have a higher HPA axis stress reactivity than males, stress might also have a bigger impact on brain function in females. For example, sex differences in HPA-axis activity may be reflected in hippocampal neurogenesis, a process that is thought to be influenced by glucocorticoids (for review, see Mirescu & Gould, 2006). Only few studies investigated the relationship between stress and neurogenesis in female rats. Acute footshock stress and exposure to fox odour were found to suppress cell proliferation in male rats, but not in females (Falconer & Galea, 2003; Shors et al., 2007). Chronic footshock stress decreased cell proliferation and/or survival in males, whereas an increase was seen in females (Westenbroek et al., 2004). However, in another study from the same lab, chronic footshock stress decreased cell proliferation and/or survival in females (Kuipers et al., 2006). Chronic high corticosterone exposure, as opposed to low CORT levels, resulted in suppression of cell proliferation and the production of new neurons in both males and females (Brummelte & Galea, 2010). Together the available data do not yet provide a clear picture on sex differences in stress and neurogenesis.

The aim of the present study was to investigate sex differences in HPA-axis regulation and sensitivity to stress-induced changes in hippocampal neurogenesis. To examine the potential role of oestradiol, females were ovariectomized and equipped with pellets resulting in constant low or high physiological levels of oestradiol throughout the experiment. We subjected males and females to a variety of stressors - from mild to more

severe and physical as well as emotional - to study differences in the neuroendocrine responses and in different aspects of hippocampal neurogenesis.

2. Method

2.1. Animals

The study was performed in adult age-matched male and female Wistar rats weighing approx. 380 and 240 g respectively at the start of the experiment. All animals were housed under a 12h/12h light-dark cycle with temperature maintained at 21±1°C. Food and water was available ad libitum. The experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

2.2. Experimental outline

We examined sex differences in the neuroendocrine response to various mild and more severe stressors, physical as well as emotional. The possible influence of different oestradiol concentrations in females was also assessed. The stress animals were exposed to the different stressors for a fixed period of 30 minutes during the first hours of the light phase, when basal activity of the HPA axis is at its lowest (e.g. de Boer & van der Gugten, 1987; Atkinson & Waddell, 1997; Windle et al., 1998). The control animals remained undisturbed throughout the experiment, except for the collection of two blood samples at the start and the end of the experiment, to verify whether basal CORT and ACTH levels are similar in stress and control animals. Furthermore, sex differences and the effects of oestradiol concentration and stress exposure on different aspects of adult hippocampal neurogenesis were assessed. The animals were divided over 6 experimental groups: control-male (CM), stress-male (SM), control-female-0.025% oestradiol (CF-low), stress-female-0.025% oestradiol (SF-low), control-female-0.1% oestradiol (CF-high), stress-female-0.1% oestradiol (SF-high). All groups consisted of 8 animals. Body weight was recorded throughout the experiment.

2.3. Surgery and oestradiol replacement

Prior to the experiments, endogenous oestradiol concentrations during the different stages of the oestrous cycle were assessed by collecting blood samples in 5 female Wistar rats. During a 5 day period, two blood samples were drawn daily from the tail, the first at lights on and the second 6 hours later (figure 1A). Based on these data, we subsequently determined the oestradiol concentration needed in the pellets to obtain physiological blood levels of oestradiol in OVX females, which would normally be present during (di)oestrus and pro-oestrus (data not shown).

For the actual experiment, female rats were bilaterally ovariectomized and received a subcutaneous estradiol-replacement pellet (β-oestradiol 3-benzoate in cholesterol, both from Sigma, St. Louis, MO, USA). The tablet-shaped pellets (approx. 1

cm in diameter and 3-4 mm thick) contained either a low concentration of estradiol (0.025%) resulting in plasma levels of oestradiol comparable to the levels observed during (di)oestrus or a high concentration of estradiol (0.1%) leading to a plasma concentration comparable to peak levels seen during proestrus. After surgery and pellet implantation the animals were allowed to recover for 2 weeks.

2.4. Stress paradigms

Novelty: To assess the HPA-axis response to a mild challenge, animals were exposed to a novel environment. The animals were transported to a testing chamber where they were placed in a new but empty standard cage without bedding material.
Fox odour: To determine the effect of a natural and purely emotional stressor on stress reactivity the animals were exposed to 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Phero Tech Inc., Vancouver, Canada), which is the main component of fox faeces and is known to provoke a HPA-axis response in rodents (Vernet-Maury et al., 1984). The day after novelty exposure the animals were transferred to the same testing room and empty cages, to prevent novelty effects, where after a vial containing gauze and 75 µl of TMT was placed in the middle of the cage.
Footshock and re-exposure stress: One week after the exposure to fox odour, rats were subjected to a paradigm normally used for classical fear conditioning, which consists of a footshock session (a severe physical stressor), and a re-exposure to the footshock box 24 hours later, during which the animals did not receive further shocks (a purely emotional stressor). During the footshock session the animals received 5 shocks, each of a duration of 3 seconds and an intensity of 0.8 mA.
Restraint stress and stress habituation: A week after the fear conditioning paradigm, we examined if male and female rats would not only respond differently to a stressor but also habituate differently following repeated exposure to this stressor. Rats were subjected to immobilization or restraint stress by enclosing them in a wire mesh restrainer fitted closely to body size for 30 minutes daily on 5 consecutive days. It was shown previously that such repeated exposure to restraint leads to rapid habituation and decrease of the HPA axis response in male rats (Ma & Lightman, 1998).

2.5. Blood sampling and analysis

For each of the stress conditions mentioned, blood samples were taken at three time points: prior to the stress exposure (baseline), directly after the 30 minutes stress exposure (peak HPA axis response) and 45 minutes after the stressor (recovery of the stress response). For the habituation stress paradigm blood was sampled only on day 1 and day 5. Blood samples of approx. 0.5 ml were drawn from the tail (Fluttert et al., 2000; Meerlo et al., 2002; Hulshof et al., 2011) and collected in pre-chilled cups containing EDTA as anticoagulant. Thereafter, the samples were centrifuged at 4°C for 15 minutes at 2600 g, and the supernatant stored at -80°C until further analysis. ACTH and CORT levels were determined by radioimmunoassays (MP Biomedicals, Orangeburg, NY, USA). To verify

whether basal CORT and ACTH levels in stressed animals were different from controls, two blood samples were drawn from the control animals parallel with the stress animals. The first sample was collected in the morning of the first day and the second on the last day of the experiment.

The samples taken on the first and the last day of the experiment were also used to determine oestradiol levels (radioimmunoassay; MP Biomedicals, Orangeburg, NY, USA).

2.6. Brain collection and immunohistochemistry

On the last day of the experiment, animals were sacrificed 2h after the end of the restraint session. Animals were deeply anaesthetized with pentobarbital and sacrificed by transcardial perfusion with saline, followed by 4% paraformaldehyde. Brains were removed from the skull, postfixed overnight and subsequently cryoprotected in a 30% sucrose solution. After freezing the brains with liquid nitrogen, 12 series of 30 μm thick coronal sections spanning the whole hippocampus (bregma -2.3 until -6.4, according to Paxinos & Watson, 2007) were cut on a cryostat microtome. Free floating sections were stored in 0.01 M PBS containing 0.1% sodium azide until further processing.

Brain sections were analyzed to establish sex differences and the effects of oestradiol and stress on different aspects of adult hippocampal neurogenesis. Cell proliferation was assessed by means of immunohistochemical staining for Ki-67, a nuclear protein that is expressed during all phases of the cell cycle, except G0 (Scholzen & Gerdes, 2000). Differentiation of new cells was examined by staining and analysis of the expression of doublecortin (DCX), a microtubule-associated protein which is found in immature neurons (Rao & Shetty, 2004; Couillard-Depres et al., 2005). Survival of new cells was assessed by staining for the exogenous thymidine analogue 5-bromodeoxyuridine (BrdU), which is incorporated into the DNA during the S phase of the cell cycle (Kee et al., 2002). To that purpose all animals received a single intraperitoneal injection with 100 mg/kg BrdU (50 mg/ml in saline, pH 7.0; Sigma, St. Louis, MO, USA). BrdU was administered 4 days before the start of the experiment, based on the notion that progenitor cells stop dividing within 1-3 days after labelling (Dayer et al., 2003). Therefore, any change in the number of labelled cells in the course of the experiment would indicate a change in survival.

For the Ki-67 and DCX staining, free-floating brain sections were treated with 0.3% H2O2 for 30 min, followed by incubation with 3% normal serum and 0.1% TritonX-100. Next, sections were exposed for 72 h to monoclonal mouse-anti-Ki-67 (1:200, Monosan, Uden, The Netherlands) or to polyclonal goat-anti-DCX (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After thorough rinsing, brain slices were treated with 3% normal serum and 0.1% TritonX-100, followed by 2 h incubation with biotinylated goat-anti-mouse for the Ki-67 staining or biotinylated rabbit-anti-goat for the DCX staining (1:400 for both antibodies, Jackson ImmunoResearch, Suffolk, UK). Subsequently, the avidine-biotin complex (ABC Elite kit, Vector Laboratories, Burlingame,

CA, USA) was added for 2 h, after which the staining was visualized with 0.2 mg/ml diaminobenzidine and 0.0033% H_2O_2.

The brain sections which were stained against BrdU were incubated consecutively for 30 minutes in 0.6 % H_2O_2 and for 2 h at 65 °C in saline sodium citrate (2x SCC) containing 50% formamide. After successive rinses with 2x SCC, 2 MHCl (37 °C for 30 min), and 0.1 M borate buffer (pH 8.5), sections were exposed to the rat-anti-BrdU primary antibody (1:800, Oxford Biotechnology, Oxfordshire, UK) overnight at 4 °C. As secondary antibody, biotinylated donkey-anti-rat antibody was used (1:400, Jackson ImmunoResearch, Suffolk, UK). Then, sections were incubated in streptavidin–horseradish peroxidase (1:200, Zymed laboratories, Invitrogen corporation, Carlsbad, CA, USA) for 2 h at room temperature, rinsed, and reacted with DAB and H_2O_2.

After the staining, sections were mounted onto glass slides for microscopic analysis. Considering their functional difference, the dorsal and ventral portions of the hippocampus were analyzed separately (dorsal hippocampus: bregma -2.3 until -4.4; ventral hippocampus: bregma -4.4 until -6.4; (Paxinos & Watson, 2007). The dorsal hippocampus is mainly associated with spatial learning and memory, while the ventral portion of the hippocampus seems to be more involved in emotional and neuroendocrine functioning (e.g. Moser & Moser, 1998). Ki-67 and BrdU positive labelled cells were counted in the subgranular zone of every 12th section of the hippocampal formation at 400-times magnification. The number of positive cells was corrected for the length of the dentate gyrus. Results are shown as number of cells per mm.

DCX immunoreactivity was measured by a method adapted from previous studies performed in our laboratory, which demonstrated that optical density measurements are a reliable method to determine changes in the number of DCX-positive cells (e.g. Dagytė et al., 2009). The optical density of the DCX staining in the granule cell layer and the inner and middle molecular layer was measured in every 12th section throughout the entire anteroposterior extent of the hippocampus. A vast majority of the dendrites of the DCX positive cells extend into the middle molecular layer (Rao & Shetty, 2004). The optical density of DCX-expression was corrected for non-specific background staining as measured in the corpus callosum. Optical density measurements were performed by image analysis (Leica Qwin, Rijswijk, The Netherlands). An average value per section was calculated for each animal.

2.7. Statistical analysis

The ACTH and corticosterone responses to different stressors were subjected to analysis of variance (ANOVA) with repeated measures to test for sex differences (M vs. F-low and M vs. F-high) and oestradiol effects (F-low vs. F-high). The relative stress-induced increases in ACTH and CORT, both as deviation from baseline and as percentage of baseline, were analysed and subjected to a one-way ANOVA to test for an effect of sex (M vs. F-low and M vs. F-high) and/or estradiol level (F-low vs. F-high). The different neurogenesis markers (Ki-67, DCX and BrdU) were first analyzed with one-way ANOVA to test for an overall stress effect across the three treatment groups (controls vs. stress).

The data were then analyzed with two-way ANOVA to test for interactions between stress (control vs. stress) and sex (M vs. F-low and M vs. F-high) or oestradiol level (F-low vs. F-high). Body weight data were analyzed in a similar way, first with a repeated measures ANOVA to test for an overall stress effect across the three treatment groups (control vs. stress), then with two-way repeated measures to test for interactions between stress (control vs. stress) and sex (M vs. F-low and M vs. F-high) or oestradiol level (F-low vs. F-high). When appropriate, post hoc Tukey tests were applied to assess differences between specific groups at specific time points. The oestradiol levels in the OVX-females at the beginning and the end of the experiment were analyzed by one-way ANOVA (F-low vs. F-high). Data in the results section and figures are expressed as means with standard errors (SEM). Statistical significance was set at $P<0.05$.

3. Results

The oestradiol levels established by the implantation of 0.1% and 0.025% oestradiol pellets were similar to the endogenous oestradiol levels in intact females during the proestrus and (di)oestrus phase of the oestrous cycle (figure 1). In the female OVX rats, the 0.025% and 0.1% oestradiol replacement pellets resulted in plasma levels of oestradiol in the range of 65 till 115 pg/ml. Oestradiol levels obtained after implantation of 0.1% pellets were significantly higher than levels of 0.025% pellets at the beginning as well as the end of the experiment (start experiment: $F_{1,30}=5.84$; $p=0.022$; end experiment: $F_{1,30}=28.81$; $p<0.001$).

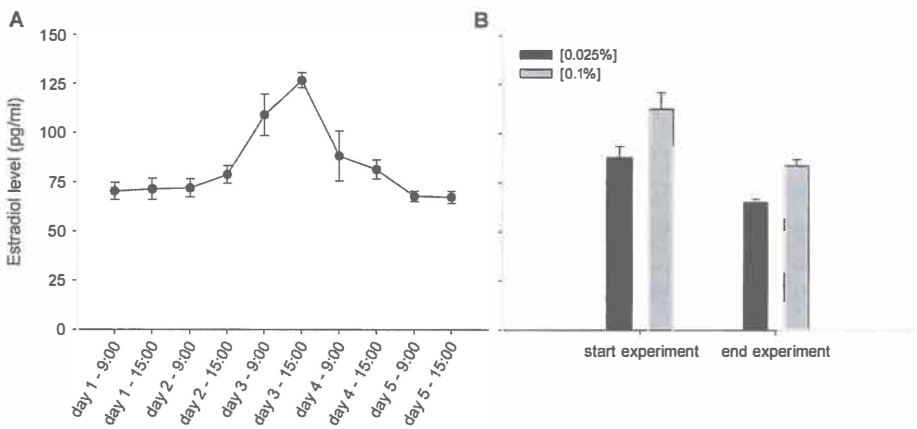

Figure 1 *Plasma levels of oestradiol across the oestrous cycle in intact female rats (panel A) and after oestradiol pellet implantation in ovariectomized rats (panel B). A) Blood samples were taken in 5 female Wistar rats to assess the oestradiol level during the different phases of the oestrous cycle. Samples were collected twice a day, at lights on and 6h later, on 5 consecutive days. B) Blood samples were collected in OVX females with oestradiol replacement pellets at the start and the end of the experiment. The 0.1% pellets resulted in high oestradiol levels comparable to the pro-oestrus phase, while the 0.025% group showed oestradiol levels similar to (di)oestrus. The oestradiol levels of the 0.1% oestradiol females were significantly higher, at both the start and the end of the experiment, than the oestradiol levels of the 0.025% female rats (start: $F_{1,30}=5.844$ $p=0.022$; end: $F_{1,30}=28.814$ $p<0.001$).*

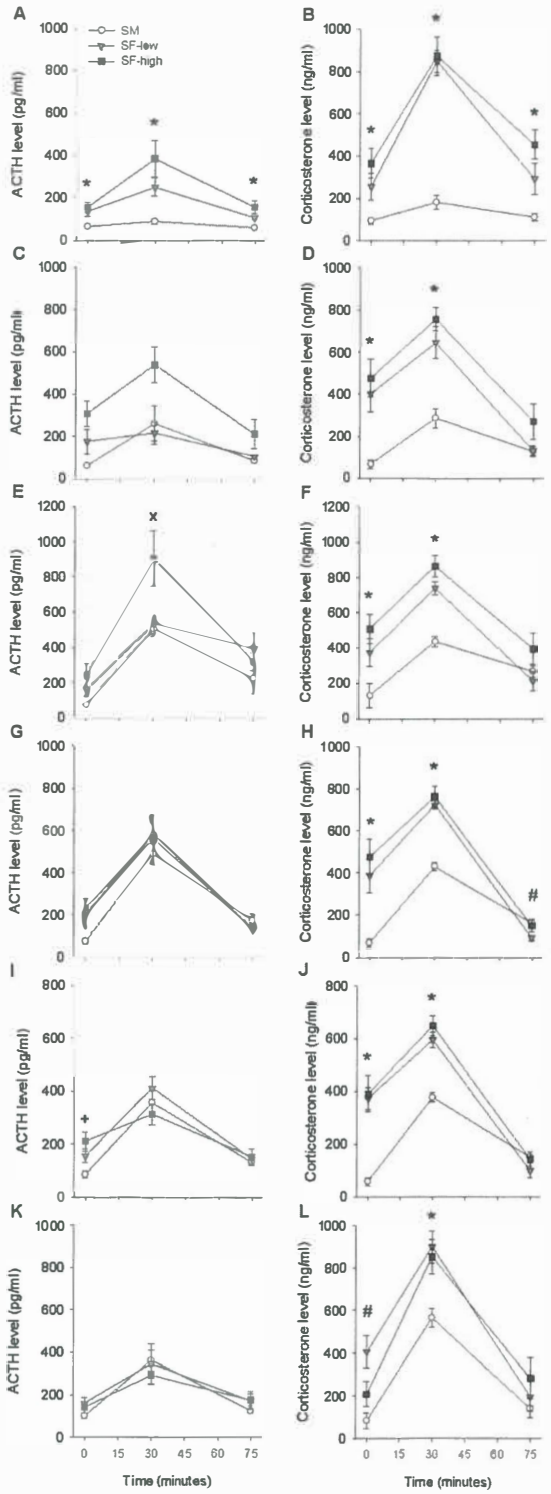

Figure 2 *Sex differences in HPA-axis response to novelty (panel A-B), fox odour (panel C-D), footshock stress (panel E-F), shock box re-exposure (panel G-H), and restraint stress (day 1: panel I-J; day 5: panel K-L). The duration of all the stressors was 30 minutes. Blood samples were collected for assessments of ACTH and CORT levels at baseline (0 minutes), after the stress exposure (30 minutes) and after 45 minutes of recovery (75 minutes). The basal CORT levels and the stress responses were significantly higher in both female groups compared to males irrespective of type of stressor. The ACTH levels were not clearly influenced by sex and significant differences were only observed during novelty exposure and the first day of restraint stress. Within females, oestradiol concentration only had a minor influence on the neuroendocrine response, with significant differences in ACTH levels during footshock and restraint stress day 1. * significant differences SM vs. SF-low and SF-high; # significant differences SM vs. SF-low; + significant differences SM vs. SF-high; × significant differences SF-low vs. SF-high.*

Body weight was significantly affected by the oestradiol level in the OVX-females, that is, growth was significantly lower in females implanted with 0.1% oestradiol pellets compared to the 0.025% group (oestradiol level x time interaction: $F_{14,420}=7.49$; $p<0.001$; data not shown). Furthermore, body weight significantly decreased in response to the stress procedure in both sexes (stress x time interaction: $F_{19,798}=8.30$; $p<0.001$; data not shown).

Basal levels of ACTH and CORT on the first and last day of the experiment were not different between the undisturbed control rats and the rats subjected to the series of stressors (data not shown). The sex differences in HPA-axis response to different stressors and the role of oestradiol herein are shown in figure 2. Overall, basal ACTH and CORT levels and the stress-induced CORT responses always seem to be higher in females compared to males, with minor differences caused by the oestradiol concentration in the two female groups.

Repeated measures analysis revealed significant differences in ACTH and CORT response during novelty exposure between males and SF-low (ACTH: sex effect $F_{1,13}=20.52$, $p=0.001$ and sex x time interaction $F_{2,26}=4.85$, $p=0.016$; CORT: sex effect $F_{1,13}=98.30$, $p<0.001$ and sex x time interaction $F_{2,26}=14.11$, $p<0.001$) and between males and SF-high (ACTH: sex effect $F_{1,13}=18.28$, $p=0.001$ and sex x time interaction $F_{2,26}=4.57$, $p=0.020$; CORT: sex effect $F_{1,13}=60.64$, $p<0.001$ and sex x time interaction $F_{2,26}=8.44$, $p=0.001$; figure 2A-B). There was no significant difference between the two female groups. The ACTH and CORT levels were significantly higher in SF-low compared to SM at all 3 time points of the response (ACTH: $p<0.05$ for baseline, stress and recovery; CORT: $p<0.05$ for baseline, stress and recovery). Also SF-high showed significantly higher HPA-axis activity than males at all 3 time points measured (ACTH: $p<0.05$ for baseline, stress and recovery; CORT: $p<0.05$ for baseline, stress and recovery).

The ACTH response to fox odour exposure was significantly different between males and SF-high (sex effect: $F_{1,14}=13.67$ $p=0.002$) and between the two female groups (oestradiol effect: $F_{1,13}=10.65$, $p=0.006$; figure 2C). Both female groups showed significant higher CORT levels than males (SF-low vs. SM: sex effect $F_{1,14}=20.71$ $p<0.001$ and sex x time interaction $F_{2,26}=14.11$, $p<0.001$; SF-high vs. SM: sex effect $F_{1,14}=73.88$, $p<0.001$ and sex x time interaction $F_{2,26}=8.44$, $p=0.001$; figure 2D). When individual time points were analyzed separately, the two female groups had significantly higher levels of CORT at baseline and at the peak of the stress response ($p<0.05$ in each case). There was no difference in CORT response between SF-low and SF-high.

The ACTH response to footshock was significantly different between males and high oestradiol females (sex effect: $F_{1,14}=8.99$ $p=0.010$; figure 2E). Furthermore, there was a significant difference between both female groups (oestradiol x time interaction: $F_{2,28}=3.73$, $p=0.036$; figure 2E). Particularly, the ACTH levels at the end of the 30-min stress exposure were significantly higher in SF-high than in SF-low ($p<0.05$). The CORT response to footshock showed a significant sex effect between males and both female groups (SM vs. SF-low: sex effect $F_{1,14}=11.22$, $p=0.005$ and sex x time interaction $F_{2,28}=7.40$, $p=0.003$; SM vs. SF-high: sex effect $F_{1,14}=29.00$ $p<0.001$ and sex x time interaction $F_{2,28}=3.56$, $p=0.042$) and a significant overall oestradiol effect (SF-low vs. SF-high: $F_{1,14}=5.23$ $p=0.038$; figure 2F). The CORT levels were significantly different in SF-

low and SF-high compared to males at baseline and directly after the 30-min stress exposure ($p<0.05$ in each case).

While ACTH levels were comparable in all groups during re-exposure stress, the CORT data revealed significant differences between males and SF-low (sex effect $F_{1,14}=34.22$ $p<0.001$ and sex x time interaction $F_{2,28}=19.35$, $p<0.001$) and between males and SF-high (sex effect $F_{1,14}=37.06$, $p<0.001$ and sex x time interaction $F_{2,28}=14.83$, $p<0.001$; figure 2G-H). The low oestradiol females had higher CORT levels than males at all 3 time points measured ($p<0.05$ for baseline, stress and recovery), while high oestradiol females exhibited higher levels at baseline and at the peak of the stress response ($p<0.05$ in each case).

To assess possible sex differences in habituation to repeated stress exposure, the animals were exposed to restraint stress on 5 consecutive days. Contrary to our expectation, neither females nor males seem to habituate to restraint. In response to the first restraint, ACTH levels were significantly different between males and SF-high (sex x time interaction $F_{2,26}=7.69$, $p=0.002$) and between the two female groups (oestradiol level x time interaction $F_{2,26}=3.95$, $p=0.032$; figure 2I). Post hoc analysis showed a significant difference between SM and SF-high only at baseline ($p<0.05$). For the two female groups post hoc comparison of the separate time points did not reveal significant differences. The CORT response to the first restraint was significantly different between males and SF-low (sex effect $F_{1,14}=66.10$ $p<0.001$ and sex x time interaction $F_{2,28}=23.11$, $p<0.001$) and between males and SF-high (sex effect $F_{1,14}=33.29$, $p<0.001$ and sex x time interaction $F_{2,28}=16.45$, $p<0.001$; figure 2J). Post hoc analysis showed that both female groups exhibited higher CORT levels at baseline and at the peak of the stress response than males ($p<0.05$ in each case).

During the last restraint session the ACTH responses were similar in all groups (figure 2K). The CORT responses, however, where significantly lower in males than in both female groups (SM vs. SF-low: sex effect $F_{1,14}=12.62$, $p=0.003$ and sex x time interaction $F_{2,28}=4.31$, $p=0.023$; SM vs. SF-high: sex effect $F_{1,13}=24.17$, $p<0.001$ and sex x time interaction $F_{2,26}=3.59$, $p=0.042$; figure 2L). The low oestradiol females demonstrated higher CORT levels than males at baseline and at the peak of the stress response, while high oestradiol females exhibited higher levels only at the peak of the response ($p<0.05$ in each case).

Besides the absolute stress levels, the relative stress-induced increases in ACTH and CORT as deviation from the baseline level and as percentage from baseline were analysed. For most stressors, the ACTH and CORT increases as deviations from baseline were not different between sexes. However, the deviations from baseline showed significant sex differences for novelty exposure and for the first restraint stress. While the ACTH and CORT increases above baseline level after novelty were higher in females (ACTH: SM vs. SF-high $F_{1,13}=6.74$, $p=0.022$; CORT: SM vs. SF-low $F_{1,13}=26.34$, $p<0.001$; SM vs. SF-high $F_{1,13}=13.18$ $p=0.003$), the deviations from baseline after restraint stress day 1 were higher in males (SM vs. SF-high $F_{1,13}=10.91$ $p=0.006$).

Also when the ACTH and CORT increases after stress exposure were expressed as percentage of baseline, there were no sex differences after most stressors. However,

significant male-female differences for the percentage increases in ACTH and CORT were found after re-exposure to the shock box and after the first restraint stress. A significant sex difference, favouring males, was observed after re-exposure stress (CORT: SM vs. SF-low $F_{1,14}$=9.70, p=0.008; SM vs. SF-high $F_{1,14}$=5.20, p=0.039) and restraint stress day 1 (ACTH: SM vs. SF-high $F_{1,13}$=21.94, p<0.000; CORT: SM vs. SF-low $F_{1,14}$=7.44, p=0.016; SM vs. SF-high $F_{1,14}$=6.88, p=0.020).

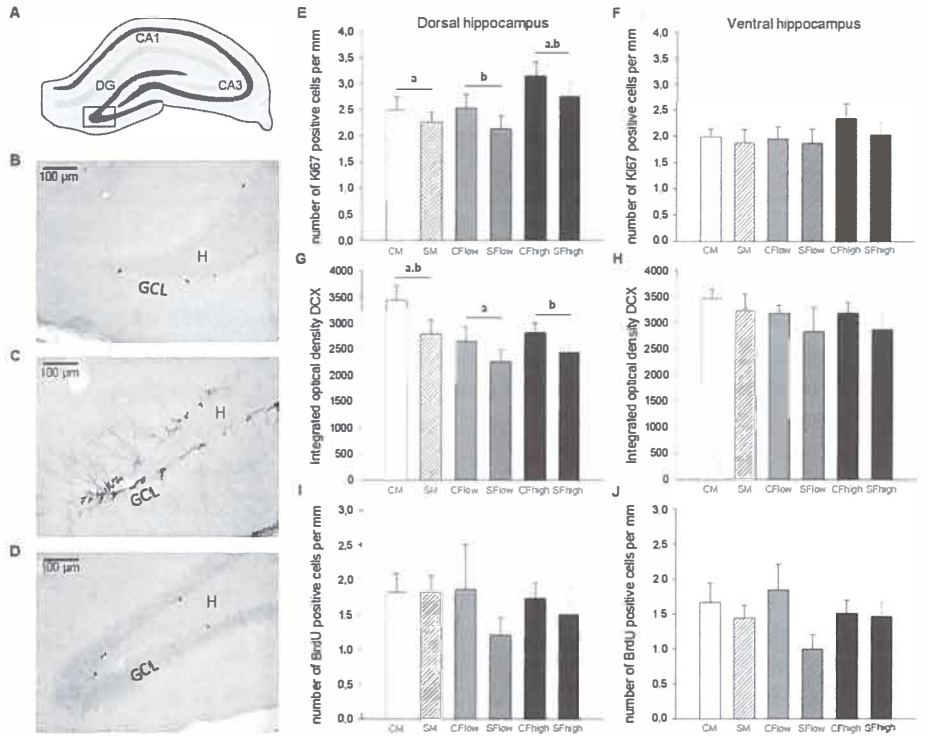

Figure 3 *Sex differences in hippocampal neurogenesis under baseline and stress conditions. Panel A gives an overview of the hippocampus and its different subregions. Panel B-D provide representative images of immune staining for Ki-67 (cell proliferation), DCX (cell differentiation) and BrdU (cell survival). The high oestradiol groups showed an increased number of Ki-67 positive cells in the dorsal hippocampus compared to both low oestradiol females and males ([a] F-high vs. M: p=0.029; [b] F-high vs. F-low: p=0.027; panel E). Adult stress did not affect the proliferation of new born cells, but showed a significant overall effect on cell differentiation (control vs. stress p=0.018; panel G). Furthermore, the integrated optical density of DCX was significantly higher in males than in females ([a] M vs. F-low: p=0.018; [b] M vs. F-high: p=0.031). Unlike cell proliferation and differentiation, cell survival was not found to be affected differently in both sexes, and was not significantly influenced by stress or oestradiol concentration (panel I and J).*

Sex differences in, and the effect of oestradiol level and stress on the proliferation, neuronal differentiation and survival of newborn cells in the dorsal and ventral hippocampus were studied by staining for respectively Ki-67, DCX and BrdU (figure 3). There was no significant effect of stress on cell proliferation in the dorsal or ventral hippocampus. In the ventral hippocampus there was no significant effect of sex and

oestradiol either. However, in the dorsal hippocampus cell proliferation was significantly affected by sex and oestradiol, since the number of Ki-67 positive cells was significantly higher in F-high compared to F-low and males (F-high vs. M $F_{1,28}$=5.28, p=0.029; F-high vs. F-low $F_{1,28}$=5.47, p= 0.027).

Cell differentiation in the dorsal hippocampus, as indicated by DCX expression, was significantly reduced by stress (overall stress effect: $F_{1,42}$=6.04, p=0.018). In addition, cell differentiation in the dorsal hippocampus was significantly affected by sex. Particularly, DCX expression was significantly higher in males than in low and high oestradiol females (M vs. F-low $F_{1,28}$=6.36, p=0.018; M vs. F-high $F_{1,28}$=5.15, p=0.031). The optical density of DCX in the ventral hippocampus was neither affected by stress nor by sex and oestradiol level.

The number of surviving BrdU-labelled cells in the dorsal and ventral hippocampus was not significantly changed by either sex, oestradiol level or stress (figure 3).

4. Discussion

In the present study, female rats showed a substantially higher basal adrenal activity and higher corticosterone levels upon exposure to stress than male rats, independent of the type of stressor. This higher adrenal activity in female rats was not substantially influenced by estradiol concentration. Despite the remarkable male-female differences in HPA-axis activity, the effects of repeated stress exposure on neurogenesis were not different between males and females. Both sexes showed a stress-induced reduction in hippocampal DCX expression, suggesting a reduced number of young neurons.

The primary aim of the present study was to investigate sex differences in stress-induced changes in neurogenesis. Although several reports described the effects of stress on neurogenesis in male rats, studies on the interaction between glucocorticoids, neurogenesis and sex or oestradiol are rather scarce. In the current study, cell proliferation assessed at the end of the stress period was not affected in either sex. However, acute and short-lasting changes in cell proliferation after specific stressors earlier on in the experiment that normalized over time cannot be ruled out. Furthermore, cell survival in both males and females remained similar during repeated stress exposure, while the differentiation of new cells to neurons was significantly decreased by stress in both sexes. The lack of a clear stress-induced change in cell proliferation is in contrast to previous findings, where 2h after footshock stress or 24h after exposure to fox odour cell proliferation was decreased in male but not female rats (Falconer & Galea, 2003; Shors et al., 2007). However, in a recent study, in which rats were exposed to low corticosterone doses, leading to levels similar to the ones observed in our males, the number of Ki-67 positive cells remained unchanged in both males and females (Brummelte & Galea, 2010). A high CORT concentration reduced cell proliferation in both sexes (Brummelte & Galea, 2010). However, in that case, the animals displayed a constant high CORT level comparable to the peak levels observed in our females, which might explain why Brummelte & Galea found a reduction in cell proliferation in the females and we did not.

In another study, chronic footshock stress decreased cell proliferation and/or survival in males, whereas it increased it in females, when multiple BrdU injections were administered during the stress period (Westenbroek et al., 2004). However, the same research group reported that administration of BrdU at a later time point during the stress period decreased cell proliferation and/or survival in females after chronic footshock stress (Kuipers et al., 2006).

We found a clear stress-induced decrease in DCX expression in both sexes, which might suggest a reduced differentiation of new cells into neurons. The literature on the effects of stress in males is rather inconsistent, with some reporting no influence of stress (e.g. Thomas et al., 2007), while others show clear stress-induced decreases in the number of new neurons (e.g. Dagytė et al., 2009). Although in the current study stress significantly reduced cell differentiation in both males and females, the substantially higher stress levels in females compared to males, did not result in a proportionally larger decrease in new neurons. However, it has been suggested that the relative increases in stress hormones are of greater physiological importance than the absolute stress levels (Rhodes & Rubin, 1999). Whereas in the present study females had consistently higher absolute levels of CORT and ACTH, the relative increases over baseline, both as percentage and deviation, were not different between males and females for most stressors. The percentage increase over baseline was higher in males after re-exposure to the shock box and after the first restraint stress.

Obviously, effects of stress and glucocorticoids are not simply determined by the plasma levels of the hormone, but also by the sensitivity to the signal in terms of receptor density. Stress-induced changes in corticosteroid receptors might play a role in the "protection" of young neurons to the higher CORT levels in females. Under basal conditions there is no clear sex difference in the number of glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus (e.g. Turner, 1992; MacLusky et al., 1996). However, Karandrea and colleagues suggested that long-term stress exposure can significantly increase MR mRNA expression in females (Karandrea et al., 2000). In the hippocampus, MRs may have a neuroprotective effect (e.g. Almeida et al., 2000). Furthermore, Cameron and colleagues reported that some hippocampal progenitor cells show MRs as soon as 24 hours after division (Cameron et al., 1993). Therefore, in the present experiment the stress exposure might have increased MR mRNA expression and, if translated into protein, either directly or indirectly protected the young neurons in the female rats.

The aforementioned sex difference in HPA-regulation, with females showing higher basal HPA-axis activity and higher stress-induced glucocorticoid levels than males, was independent of oestradiol level and stressor. The basal and stress-induced levels of ACTH and CORT were essentially similar in the two female groups with different concentrations of oestradiol replacement. These findings are in line with most of the literature (e.g. Kitay, 1961; Le Mevel et al., 1979; Aloisi et al., 1994; Atkinson & Waddell, 1997; Rivier, 1999; Shors et al., 1999), although not all (Raps et al., 1971; Atkinson & Waddell, 1997).

Unlike the rather consistent male-female differences in HPA-axis regulation, the findings on sex differences in neurogenesis are more variable. Our study indicates basal sex differences in different aspects of hippocampal neurogenesis, particularly cell proliferation and differentiation. Cell proliferation was significantly higher in females with high levels of circulating oestradiol compared to females with low oestradiol levels and to males. However, the differentiation of new cells to neurons, as indicated by the expression of DCX, was higher in males compared to both low and high oestradiol females. There was no significant male-female difference or oestradiol-induced change in the survival of new cells labelled with BrdU. Although similar findings on cell proliferation and cell survival are reported (Tanapat et al., 1999), in some papers general male-female differences in (Falconer & Galea, 2003; Shors et al., 2007; Brummelte & Galea, 2010) and estradiol effects on (Tanapat et al., 2005; Barha et al., 2009) different aspects of neurogenesis were absent. Whereas, to our knowledge, sex differences, or an absence thereof, in DCX expression in adulthood were not yet reported, a study in young 21 day old rats showed a similar male-female difference, favouring males, as observed in the present paper (Oomen et al., 2009).

Similar to the present study, Tanapat and colleagues observed higher cell proliferation levels in intact females than males, but similar numbers of surviving cells, and therefore suggested that oestradiol induces a transient increase in the pool of new born cells (Tanapat et al., 1999). This was supported by differences in BrdU-positive cell numbers after 4, 7 and 14 days, but not 21 days of survival between females injected with BrdU during proestrus or oestrus (Tanapat et al., 1999), showing that high oestradiol, as in our study, results transiently in higher cell numbers.

In contrast to the possible role of circulating estradiol in cell proliferation, cell differentiation does not seem to be influenced by estradiol. The lower basal expression of DCX in females when compared to males might be related to the higher basal CORT levels in females. Furthermore, testosterone may play a role in the observed sex difference. Androgen receptors have been reported in the hippocampus, but appear to be rare or absent in the dentate gyrus (Simerly et al., 1990). Despite this apparent absence of androgen receptors in the dentate gyrus, testosterone was found to up-regulate cell survival in the hippocampus of gonadectomised male rats (Spritzer & Galea, 2007). Although the influence of testosterone on DCX expression was not studied in rodents, Balthazart and colleagues reported an increased DCX expression in the HVC nucleus, a song control nucleus in song birds, in response to testosterone administration in canaries (Balthazart et al., 2008). Hence, both CORT and testosterone might play a role in the higher DCX expression currently observed in the male rats.

Despite the suggested functional difference between the dorsal (associated with learning and memory) and the ventral portion (involved in emotional and neuroendocrine functioning) of the hippocampus (Moser & Moser, 1998), only few of the cited studies investigated the effects separately for these regions. In the present study, sex differences and stress-induced changes in cell proliferation and differentiation were apparent in the dorsal hippocampus, while neurogenesis was not significantly altered in the ventral hippocampus. The functional implications of the observed sex differences and oestradiol effects in the dorsal hippocampus are unknown.

Part of the aforementioned inconsistency in sex differences in basal and stress-induced changes in neurogenesis in our study and literature in general, may be related to methodological issues like, for example, the timing and manner of oestradiol administration. Besides the obvious influence of oestradiol concentration, the interval between OVX and oestradiol replacement might play a role. Ovariectomy was found to reduce dendritic spine density in CA1 pyramidal cells of the hippocampus and decrease cell proliferation, while increasing cell death in the dentate gyrus (e.g. Gould et al., 1990; Tanapat et al., 1999). Although acute oestradiol replacement seems to annul most of these changes, the inability of oestradiol to increase cell proliferation 4 weeks after OVX suggests a reduced ability of progenitor cells to respond to oestradiol, resulting from a reduction in oestrogen receptors (Gould et al., 1990; Tanapat et al., 1999; Tanapat et al., 2005; Barha & Galea, 2010). Further variance in literature might be caused by the administration of oestradiol in either a cyclic or continuous, chronic way. In the present study, we equipped the females with low or high physiological oestradiol pellets, thereby obtaining constant oestradiol levels, to be able to study the responses to the different stressors during the same phase/oestradiol level. However, since oestradiol treatment was found to either up-regulate or down-regulate oestrogen receptors in the hippocampus (Weiland et al., 1997; Jin et al., 2005), the constant and chronic administration of oestradiol in the present study might have influenced the outcome. Although Tanapat and colleagues showed no clear difference between cyclic or continuous oestradiol replacement on cell proliferation when oestradiol was administered for a period of 3 weeks (Tanapat et al., 2005), it is not excluded that the cyclic availability of estradiol, rather than the absolute levels, are important for the development of "normal" sex differences.

In summary, the present study confirms that female rats in general have a higher HPA-axis activity and higher corticosterone levels under conditions of stress than males, a difference that appears to be independent of the oestradiol concentration in females. Secondly, despite male-female differences in HPA-axis activity, the stress-induced decrease in cell differentiation was not different between the sexes. Therefore, this study does not support the hypothesis that differences in HPA-axis regulation are important mediators of sex differences in brain plasticity and sensitivity to psychopathology.

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6.

General discussion

Edward Sharpe & The Magnetic Zeros - Home

Both early life stress and sex are thought to play an important role in the individual differences in adult stress sensitivity and susceptibility to stress-related disorders. Adverse early life experiences as well as female sex were found to constitute a risk factor for the development of for example depression. The primary aim of this thesis was to investigate the long-term (functional) consequences of maternal separation and to study the male-female differences in stress-related alterations in neuroplasticity and sleep. Below the main findings of this thesis will be summarized and discussed. Furthermore, possible caveats in the experiments and the functional implications of the findings will be described.

1. Long-term consequences of early life stress

The first part of the thesis addressed the long-term consequences of maternal separation (MS) during early postnatal life in adulthood. The first two chapters of this thesis show that only minor differences exist in adult stress responsivity between maternally separated rats and controls. Although basal HPA-axis activity appeared to be slightly higher in MS animals, the neuroendocrine response to stress was similar in both groups, independent of the type or intensity of the stressor the animals were exposed to in adulthood (chapter 3). Similarly, the autonomic neural regulation of heart rate exhibited little difference between MS and control rats (chapter 2). Despite minor differences in the adult heart structure of MS rats when compared to controls, the basal adult sympathovagal balance and intrinsic heart rate were similar in both groups. Also under an acute challenging condition, the adult cardiac autonomic responsiveness was not influenced by maternal separation. Although maternal separation had no long-term effects on stress reactivity, MS rats showed significant differences in hippocampal neurogenesis and impaired cognitive functioning in a novel object recognition task (chapter 3). Maternal separation significantly reduced cell proliferation in the ventral portion of the hippocampal dentate gyrus when compared to controls, while MS and control animals showed no difference in cell survival. The proportionally higher survival rate relative to the proliferation rate in MS rats compared to controls might suggest that the pool of newborn cells in MS rats is more robust. Although the ventral hippocampus is associated with amongst others emotional functioning, there was no significant effect of MS on anxiety-like behavior. Altogether these data show that maternal separation, for 3h per day during the first two postnatal weeks, induces only minor changes in hippocampal plasticity and cognitive functioning and minimally influences adult stress sensitivity.

Considering that the stress associated with the mother-litter separation occurred during a crucial ontogenetic period and therefore is likely to disrupt normal development, the lack of (major) long-lasting alterations in adult maternally separated animals was rather unexpected. Additionally, our model was based on the initial studies on maternal separation, which suggested that MS permanently alters adult neuroendocrine and behavioral stress reactivity (Plotsky & Meaney, 1993). However, in contrast to general belief, the current data indicate that interfering with the mother-infant relationship during early development does not necessarily lead to persistent changes in stress sensitivity and emotionality in the adult offspring, and therefore cannot be seen as a model for depression.

The absence of a generalized response to maternal separation might be related to a genetic resilience of certain strains of rats or mice, to differences in maternal care between strains, or to a complex combination of the two. Some recent studies in mice indicated clear strain differences in the sensitivity to early life stress, showing strain-specific alterations in for example cognitive functioning and anxiety-like behavior in response to maternal separation (Binder et al., 2011; Mehta & Schmauss, 2011). Moreover, two previous studies showed clear within-strain variability. In a study by Sánchez and colleagues it was suggested that only about 20 percent of MS rats displayed HPA-axis hyperresponsivity in adulthood, while even fewer MS animals exhibited increased anxiety-like behavior (Sánchez et al., 2001). Another study, focusing on MS-induced alterations in cognitive functioning throughout life, proposed that approximately 40 percent of the animals was resistant to the negative effects of maternal separation (Oitzl et al., 2000). Although these two studies employed a 24h MS protocol, the observed resilience might apply to maternal separation models in general.

The variability in the outcome of MS experiments might in part be related to variations in maternal care, which in itself may again be determined by genetic factors. The balance between the prolonged absence of the mother and the subsequent maternal care an individual receives may be essential for the (long-term) outcome of MS offspring. Although in the studies presented in this thesis maternal behavior was not examined, Macri and colleagues (2004) previously showed that maternal separation induces (compensatory) active nursing, consisting of licking, grooming and arched back nursing, following upon reunion. Furthermore, a general up-regulation in maternal care levels throughout the rest of the day was shown in maternally separated animals when compared to undisturbed controls (Macri et al., 2004). Maternal care was found to influence the development of the neural systems involved in, amongst others, behavioral and neuroendocrine stress reactivity (Champagne et al., 2003). Naturally occurring high maternal care resulted in less anxious and stress reactive offspring when compared to offspring of low maternal care mothers. Therefore, the enhanced maternal care and the prolonged mother-litter separation appear to be opposing factors. The naturally occurring variations in maternal care between mothers and the within-litter variation in maternal care (Champagne et al., 2003) might explain the variation in outcome in the MS offspring. While in some animals the received maternal care might compensate for the prolonged absence of the mother, in others it might not. Therefore, the absence of clear long-term alterations in the adult offspring in the present studies might be related to a genetic resilience of Wistar rats to MS, possibly connected to high maternal responsivity, as well as a MS-induced increase in maternal care. Whether indeed an increase in maternal care is responsible and counteracted or even prevented the negative impact of maternal separation, could be studied by submitting the offspring of high and low active maternal care mothers to a maternal separation protocol. While in high maternal care offspring the negative consequences of maternal separation are likely to be prevented by the increase in maternal care, the low maternal care mothers may not be able to fully compensate for the prolonged absence of the mother, even if an increase in contact time with the offspring would occur.

It was previously suggested that alterations in stress reactivity induced by early life events might serve an adaptational purpose (Meaney, 2001). Environmental factors and conditions were found to influence maternal behavior, which in turn affects the development of the offspring. So, by altering the maternal care the mother can "program" the individual's stress reactivity as such that it will be appropriate for the environmental conditions. Under adverse environmental circumstances, e.g. presence of predators and social competition, heightened neuroendocrine and behavioral stress reactivity might form an environmental advantage. However, it is questionable whether in case of maternal separation one can speak of a general adaptive response since only part of the animals submitted to maternal separation display an altered stress reactivity. Furthermore, one might wonder why the mother would try to completely compensate for the prolonged absence by increasing active maternal care if it would serve an adaptational role.

However, to obtain an unambiguous view of a possible adaptational significance of alterations induced by an adverse environment, this should be studied in a more natural setting, which does not involve human handling. Although a previous study attempted this by varying the spatial and temporal food availability during the first 8 postnatal days, a possible caveat might be the food restriction, resulting from the temporal availability of food, in one of the experimental groups (Macri & Würbel, 2007). It has been reported that maternal food restriction in itself may affect the offspring's HPA-axis reactivity (Léonhardt et al., 2002). Therefore, instead of differences in food availability per se, it might be interesting to alter the pressure or required activity to obtain enough food, by e.g. exercise or operant behavior.

2. Sex differences in adult stress sensitivity

Another factor thought to underlie variations in stress sensitivity between individuals is sex, which was the focus of the second part of this thesis. In the last two experimental chapters, the sex differences in neuroendocrine stress reactivity and in stress-induced alterations in sleep and neurogenesis were studied. In both studies the females were ovariectomized and equipped with replacement pellets resulting in constant physiological estradiol levels. In general, females showed higher basal and stress-induced adrenal activity when compared to males, independent of the nature and intensity of the stressor (chapter 4 & 5). Furthermore, there was no substantial difference in HPA-axis activity between females with low or high estradiol levels, corresponding to respectively the (di)estrus and proestrus phase of the estrous cycle (chapter 5). Chapter 4 shows that there were no significant sex differences in sleep-wake architecture under baseline conditions or following an acute challenge, i.e. a physical (footshock) and emotional (re-exposure) stressor. In contrast, sex was found to significantly influence different aspects of hippocampal neurogenesis. Whereas cell proliferation was significantly higher in the high-estradiol females compared to low estradiol females and males, the expression of DCX, a marker for cell differentiation, was higher in male rats than in female rats, independent of estradiol level (chapter 5). While the differences in cell proliferation are likely caused by estradiol, cell differentiation appears to be unaffected by estradiol level. It would be interesting to

examine whether, as previously suggested, the higher baseline glucocorticoid levels in females or the presence of testosterone in males may be responsible for the sex difference observed in cell differentiation.

Prolonged stress exposure significantly decreased cell differentiation, while other aspects of neurogenesis, cell proliferation and cell survival, remained unaffected. Thus, while clear sex differences were observed in stress responsivity, this was not paralleled by the stress-induced changes in sleep and neurogenesis, which were rather small and largely similar in both sexes. Therefore, this thesis does not support the hypothesis that sex differences in HPA-axis reactivity trigger sex differences in brain plasticity or sleep. It rather suggests, opposed to the generally stated heightened sensitivity to stress and stress-related pathologies in females, that our female Wistar rats might have been more resistant to stress-induced alterations. Similarly, Galea and colleagues reported that female rats do not show chronic restraint stress-induced dendritic atrophy of the CA3 pyramidal neurons seen in male rats, despite higher CORT levels in females (Galea et al., 1997).

Stress resilience appears to be mediated by a multitude of factors, including different components of the stress response systems itself (for review, see Charney, 2004). Besides genetic variants in, for example, the corticosteroid receptors MR and GR (DeRijk & de Kloet, 2008), several other factors like neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF) and dehydroepiandrosterone (DHEA) are suggested to be involved in the individual differences in stress resilience or vulnerability. One of the factors which might specifically play a role in the sex difference in the resistance to stress-induced alterations in neurogenesis observed in the present data is the adrenal steroid DHEA, which, like corticosterone, is secreted in response to ACTH. An in vitro study reported that the adrenals of female rats produced significantly higher levels of DHEA after incubation with pregnenolone, one of the precursors of DHEA, than the adrenals of males (Ali Askari, 1970). Previous studies suggested that DHEA may have anti-glucocorticoid and neuroprotective properties (e.g. Kalimi et al., 1994; Kimonides et al., 1998; Bastianetto et al., 1999). Therefore, possible sex differences in the stress-induced DHEA levels might explain the similar stress-induced alterations in neurogenesis in male and female rats. Future studies should investigate whether female rats indeed exhibit increased resistance to stress-induced alterations and whether DHEA or other mechanisms might be involved.

Considering that genetic influences as well as hormonal modulation by estrogens and androgens contribute to the development of sex differences, studying sex differences and the underlying mechanisms is rather complex. The present experiments did not investigate the possible influence of progesterone and/or testosterone, but were restricted to the role of estradiol. One aspect of our experimental paradigm that may have influenced the outcome of the studies is the estradiol replacement resulting in constant levels of estradiol rather than the naturally occurring cyclic presence of the hormone. Although a previous study reported no significant differences between cyclic or continuous estradiol replacement on specifically cell proliferation (Tanapat et al., 2005), it cannot be excluded that the cyclic presence of estradiol might be essential for finding "normal" sex differences. Another aspect of our experimental setup that may have influenced the results is the fact that ovariectomy reduces the levels of multiple sex hormones, but only estradiol levels were

restored. Since, as briefly mentioned in the general introduction, progesterone can significantly influence neuroplasticity and sleep-wake behavior, one might argue that in the present studies the reduction of progesterone might have altered the normally present sex differences. However, a recent study suggested that the adrenals rather than the ovaries are the mean source of progesterone during the estrous cycle (Flores et al., 2008).

3. Translation to humans

At the basis of the studies investigating the influence of maternal separation and sex on stress sensitivity are epidemiological studies indicating adverse early experiences and sex as risk factors for the development of psychopathologies like depression (e.g. Weissman et al., 1996; Felitti et al., 1998). Alterations in various systems and processes are implicated in the development of depression, including monoaminergic systems, the immune system, sleep, the HPA-axis and neuroplasticity (e.g. Charney, 1998; Jacobs et al., 2000; Holsboer, 2001; Miller et al., 2009; Dobos et al., 2010; Lucassen et al., 2010). In the current thesis we focused specifically on possible differences or alterations in the stress response systems, sleep and neurogenesis. Sleep disturbances and hyperactivation of the HPA-axis are commonly observed in depressive patients. A possible role for altered neuroplasticity and/or neurogenesis in depression was suggested based on clear structural changes of the hippocampus, including a reduction in hippocampal volume, in depressive patients, and on animal studies showing a stress-induced decrease in neurogenesis and an up-regulation thereof after antidepressant treatment (Kempermann & Kronenberg, 2003). Although it was previously demonstrated that the adult human brain also exhibits neurogenesis, the relevance of the relatively small numbers of new born neurons has been questioned (Eriksson et al., 1998; Cameron & McKay, 2001). On the other hand, it was recently suggested that even a small population of new neurons might impact hippocampal function (Snyder & Cameron, 2011). This was mainly based on studies showing that 1) a single granule neuron can trigger multiple CA3 pyramidal cells (Henze et al., 2002), 2) new born cells are more likely to be activated by hippocampal-dependent behaviors than older granule neurons (Ramirez-Amaya et al., 2006; Snyder et al., 2009) and 3) that the stimulation of even a single cortical neuron could alter brain state and evoke whisker movement (Brecht et al., 2004; Li et al., 2009).

As mentioned in the general introduction, the early exposure to stressful life events can have detrimental effects during the early postnatal development. Besides child neglect and abuse, the exposure of premature infants to synthetic glucocorticoids as a treatment for respiratory conditions might also have a great impact on brain development (e.g. Murphy et al., 2001). The absence of major effects in the maternally separated animals in the present studies might be related to an increase in maternal care. Such an interaction between early life stress and parental care also seems to exist in humans. Placing infants who were raised in an orphanage in a family reversed certain negative effects, including some aspects of impaired cognitive functioning, when intervention occurred within a critical time window (Bos et al., 2010). However, in abused and neglected children it is not likely that a similar compensation in care will occur. Therefore, it is of keen interest to

study 1) the potential compensating influences of maternal care and 2) the consequences of early life stress in absence of this compensational care.

Sex was also suggested to play a prominent role in the individual differences in stress sensitivity and the development of for example stress-related pathologies and sleep disorders. Women show a higher prevalence of depression and report lower sleep quality than men. In line with this, the data in this thesis clearly showed a higher stress responsivity in female rats when compared to males. However, this was not accompanied by increased stress-induced alterations in neurogenesis and sleep, which suggest that females might be more resistant to stress-induced alterations. Therefore, these data do not support the hypothesis that a higher prevalence of sleep complaints in women is a consequence of sex differences in stress sensitivity. Instead, it was recently suggested by Mong and colleagues that the differences in the perception of sleep quality between men and women might partly be associated with mood (Mong et al., 2011).

Epilogue

This thesis was aimed at investigating the impact of early life stress and sex on adult stress sensitivity. Altogether, the data presented in this thesis indicates that interfering with the mother-infant relationship during early development does not by definition lead to major long-lasting alterations in the adult MS offspring. Additionally, although clear sex differences were reported in the HPA-axis reactivity, this was not paralleled by stress-induced changes in sleep and neurogenesis, suggesting that, in contrast to common belief, female Wistar rats might be more resistant to the influence of stress.

The actual development of psychopathology and disease is presumably dependent on a complex interplay between genes, sex, and the pre- and postnatal environment. For instance, genetic variation or polymorphisms of the serotonin transporter (5-HTTLPR) or the D4 dopamine receptor were associated with a genetic vulnerability for increased anxiety and stress responsivity in human subjects (e.g. Gunthert et al., 2007; Armbruster et al., 2009). Furthermore, prenatal stress, often performed by submitting pregnant female rats to restraint stress, was found to increase stress reactivity and anxiety and impair cognitive functioning in the adult offspring (e.g. Henry et al., 1994; Vallée et al., 1997; Vallée et al., 1999). Therefore, besides focusing on the effects of one of these factors, the interaction of different factors should help to gain insight in the development and course of disorders like depression.

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Nederlandse samenvatting

Achtergrond

Het onderzoek beschreven in dit proefschrift richt zich op de mogelijke rol van postnatale stress, oftewel blootstelling aan stress vroeg in het leven, en sekse op adulte stress gevoeligheid. Stress is de respons van het lichaam op een potentieel bedreigende situatie. Belangrijke aspecten van deze respons zijn de activatie van het sympathische bijniermerg systeem (sympatho-adrenomedullary of SAM systeem) en de hypothalamus-hypofyse-bijnier as (hypothalamic-pituitary-adrenal of HPA-as). Hoewel de acute respons van deze systemen essentieel is voor normale stress adaptatie, lijkt chronische en overmatige blootstelling aan hoge stresshormoonspiegels schadelijk te zijn en bij te dragen aan de ontwikkeling van allerlei fysiologische aandoeningen en stemmingsstoornissen.

Twee factoren die een sterke invloed lijken te hebben op de gevoeligheid voor stress en stressgerelateerde ziektes in volwassen individuen zijn het geslacht van dit individu en de opgroeicondities tijdens de vroege ontwikkeling. Epidemiologische studies hebben aangetoond dat sekse en negatieve postnatale invloeden, zoals verwaarlozing of misbruik van kinderen of het verlies van een ouder, belangrijke risicofactoren vormen voor het ontstaan van psychopathologieën als depressie op latere leeftijd. De blootstelling aan stressvolle gebeurtenissen tijdens de vroege levensfase is wellicht nog schadelijker dan tijdens volwassenheid, aangezien dit mogelijk interfereert met de normale (hersen)ontwikkeling tijdens deze gevoelige postnatale periode.

Invloed van postnatale stress op stressgevoeligheid

Het eerste deel van dit proefschrift richt zich op de invloed van maternale separatie (MS), een veelgebruikt model voor postnatale stress, op de gevoeligheid voor stress later in het leven. Hiertoe werden tijdens de eerste 2 weken na de geboorte rattenpups dagelijks 3 uur van hun moeder gescheiden terwijl controle nesten ongemoeid werden gelaten. Na deze eerste 2 weken konden zowel MS- als controle nakomelingen ongestoord opgroeien tot een volwassen leeftijd van 3 maanden. De volwassen nakomelingen werden vervolgens blootgesteld aan verschillende stressoren om het effect van MS op volwassen autonome en hormonale stressreactiviteit te onderzoeken. Tegen de verwachting in bleek MS nauwelijks effect te hebben op adulte stressreactiviteit. In de eerste studie werd gevonden dat de autonome cardiovasculaire reactiviteit in controle dieren en MS dieren nagenoeg gelijk was. Er werden weliswaar kleine verschillen gevonden in de hartstructuur in de MS-ratten ten opzichte van de controledieren, maar er was geen verschil in de basale sympathovagale balans of in de autonome cardiale respons op acute stressvolle condities (hoofdstuk 2). Ook de hormonale HPA-as respons was nagenoeg hetzelfde in de MS- en controlegroep, onafhankelijk van de aard of intensiteit van de stressor (hoofdstuk 3).

Ondanks de afwezigheid van duidelijke verschillen in de adulte stressreactiviteit, veroorzaakte MS significante veranderingen in neurogenese en cognitie (hoofdstuk 3). Neurogenese, een proces waarbij nieuwe zenuwcellen worden aangemaakt, vindt in het volwassen brein met name plaats in de hippocampus, een hersengebied dat een belangrijke rol speelt bij de regulatie van emoties, stressresponsen en geheugenprocessen. Een

stressgeïnduceerde verstoring van neurogenese zou dan ook een rol kunnen spelen bij het ontstaan van cognitieve problemen en psychopathologie. Hoewel in onze studie de aanmaak van nieuwe cellen in de (ventrale) hippocampus significant verminderd was in MS ratten ten opzichte van de controle dieren, was er geen verschil in de overleving van nieuwe cellen tussen beide experimentele groepen. Dit suggereert dat MS een kortdurend effect heeft op de nieuwe celpopulatie. Hoewel er minder nieuwe cellen worden aangemaakt zijn deze wellicht robuuster, hetgeen er voor zou kunnen zorgen dat een groter aantal van deze nieuwe cellen overleeft. Verder vertoonden MS-dieren een verminderd cognitief functioneren in een test voor objectherkenning (*novel object recognition task),* terwijl een andere geheugentest (*social recognition task*) geen verschil liet zien tussen beide groepen. Al met al suggereren onze bevindingen dat maternale separatie alleen minimale veranderingen induceert in adulte stress reactiviteit en hersenplasticiteit en een beperkte invloed heeft op het functioneren van het brein.

De afwezigheid van (duidelijke) effecten in adulte MS-dieren was onverwacht, mede ook vanwege de diverse publicaties waarop de huidige studies waren gebaseerd. In tegenstelling tot de in de literatuur gesuggereerde invloed van MS, blijkt uit de huidige data dat de verstoring van de moeder-pup relatie tijdens de ontwikkeling niet noodzakelijkerwijs resulteert in een verhoogde stressgevoeligheid of veranderde emotionaliteit. Een mogelijke verklaring voor het ontbreken van deze permanente veranderingen in MS-dieren, zou kunnen worden gezocht in de genetische achtergrond van onze proefdieren. Recente studies toonden aan dat er duidelijke verschillen zijn in de gevoeligheid voor postnatale stress tussen muizenstammen. Echter, het ontbreken van een algemene respons na MS zou ook verband kunnen houden met de (individuele) variatie in maternale zorg. Een recente studie toonde aan dat de moederdieren na MS een compensatoire verhoging in de maternale zorg laten zien gedurende de rest van de dag. Bovendien is duidelijk gebleken dat variaties in maternale zorg van invloed kunnen zijn op de ontwikkeling van neuronale systemen die betrokken zijn bij de gedragsmatige en neuroendocriene stressreactiviteit. Veel actieve maternale zorg resulteert in minder angstige en minder stressgevoelige nakomelingen ten opzichte van nakomelingen die minder actieve zorg ontvangen. Daarom zou de balans tussen de langdurige afwezigheid van de moeder en de daarop volgende maternale zorg weleens bepalend kunnen zijn voor de consequenties van MS op de lange termijn. Verder onderzoek is nodig om na te gaan of een verhoging van de maternale zorg inderdaad de mogelijk negatieve gevolgen van MS kan voorkomen en of dat inderdaad een verklaring is voor de geringe effecten van MS in de door ons gebruikte rattenstam.

Sekseverschillen in stress gevoeligheid

Het tweede deel van dit proefschrift richt zich op sekseverschillen in stressgevoeligheid. Alle vrouwelijke ratten werden na ovariëctomie voorzien van estradioltabletten die zorgden voor constante, fysiologische estradiolniveaus om te voorkomen dat de cyclische verandering in sekshormonen tijdens de oestruscyclus de uitkomst van de experimenten zouden beïnvloeden. Vervolgens werden zowel vrouwelijke als mannelijke ratten blootgesteld aan diverse stressoren om de sekseverschillen in neuroendocriene

stressreactiviteit te bestuderen. In het algemeen lieten vrouwelijke ratten een hogere basale en stressgeïnduceerde bijnieractiviteit zien ten opzichte van mannetjes, onafhankelijk van de aard of intensiteit van de stressor, (hoofdstuk 4/5). Verder was er geen verschil in HPA-as activiteit tussen vrouwtjes met een lage of hoge estradiol spiegel.

In hoofdstuk 4 werd vervolgens ook gekeken naar sekseverschillen in slaap en in stressgeïnduceerde slaapverstoringen. Veranderingen in slaap-waak patronen kunnen een belangrijke rol spelen in het ontstaan van depressie en de individuele gevoeligheid hiervoor. Echter, ondanks de duidelijke sekseverschillen in hormonale stressreactiviteit, bleken de stressgeïnduceerde veranderingen in slaap uiterst klein en bovendien grotendeels gelijk in beide seksen. Er waren geen significante sekseverschillen in het slaap-waakpatroon onder basale condities en evenmin na blootstelling aan een fysieke stressor (een milde elektrische schok of *footshock*) of emotionele stressor (hernieuwde blootstelling aan de schokkamer zonder hernieuwde schok, ook wel *re-exposure* genoemd). Footshock stress resulteerde in beide seksen in een kortdurende onderdrukking van slaap, welke werd gevolgd door een compensatoire verhoging van de slaapdiepte, weerspiegeld in een verhoging van EEG slow wave activity (SWA). Om de verloren slaap in te halen lieten de mannetjes ratten vervolgens ook nog een beperkte toename in slaapduur zien ten opzichte van hun eigen baseline. Overigens was de totale hoeveelheid slaap daarbij niet significant hoger dan bij de vrouwelijke dieren. De hernieuwde blootstelling aan alleen de stress context de daaropvolgende dag (re-exposure) beïnvloedde het slaap-waakgedrag nauwelijks, met alleen een kleine verhoging in de slaapintensiteit (SWA) direct daarna in beide seksen. Kortom, ondanks een sterkere neuroendocriene activiteit en reactiviteit in vrouwelijke ratten, leidt acute blootstelling aan stress niet tot duidelijke verschillen in slaap tussen de seksen.

Duidelijke sekseverschillen werden wel gevonden in hippocampale neurogenese (hoofdstuk 5). Terwijl de vrouwtjes met een hoge estradiol spiegel een verhoogde cel proliferatie lieten zien ten opzichte van laag estradiol-vrouwtjes en mannetjes, bleek de celoverleving gelijk te zijn in beide seksen. Dit suggereert dat (hoog) estradiol een tijdelijk effect heeft op de nieuwe cellen. De expressie van het eiwit doublecortin, een marker voor de differentiatie van nieuwe cellen tot neuronen, was hoger in mannetjes dan vrouwtjes ratten, onafhankelijk van het estradiolniveau in de vrouwtjes. Dit sekseverschil zou veroorzaakt kunnen worden door de aanwezigheid van testosteron in de mannetjes of door de hogere basale corticosteronniveaus in de vrouwtjes. Blootstelling aan een stressprotocol bestaande uit verschillende stressoren was alleen van invloed op celdifferentiatie. Ondanks de eerder vermelde hogere neuroendocriene respons in vrouwtjes, was de door stress geïnduceerde vermindering in celdifferentiatie vergelijkbaar in beide seksen. Samenvattend laten de huidige gegevens zien dat ondanks een veel hogere neuroendocriene stressreactiviteit in vrouwelijke ratten, de stressgeïnduceerde veranderingen in slaap en hippocampale neurogenese niet erg verschillen tussen mannetjes en vrouwtjes. De huidige bevindingen leveren geen duidelijke ondersteuning voor de hypothese dat sekseverschillen in HPA-as activiteit leiden tot sekseverschillen in slaap en hersenplasticiteit.

Epiloog

Dit onderzoek richtte zich op de invloed van zowel postnatale stress als de rol van sekseverschillen op de volwassen stress-gevoeligheid. De huidige studies tonen aan dat, in weerwil van de algemeen heersende opinie, de postnatale verstoring van de moeder-pup relatie niet noodzakelijkerwijs de stressreactiviteit en emotionaliteit van de adulte nakomelingen beïnvloedt. Het tweede deel van dit proefschrift toonde aan dat ondanks een hogere neuroendocriene stressreactiviteit in vrouwtjesratten, de stressgeïnduceerde veranderingen in slaap en neurogenese vergelijkbaar waren in beide seksen. Dit suggereert een grotere resistentie tegen de invloed van stress hormonen in de vrouwelijke Wistar ratten.

De daadwerkelijke ontwikkeling van psychopathologische aandoeningen is waarschijnlijk het resultaat van een uiterst complexe interactie tussen de genetische achtergrond, sekse, pre- en postnatale ontwikkelingsfactoren en stresservaringen in het latere leven. Genetische variatie in bijvoorbeeld de serotonine-transporter of dopamine-receptor, maar ook prenatale stress wordt geassocieerd met een verhoogde stressreactiviteit en angst. Gezien de rol van verschillende factoren in het ontstaan van aandoeningen als depressie, zou er ook naar de interactie van deze factoren moeten worden gekeken om meer inzicht te krijgen in de ontwikkeling en het verloop van deze aandoeningen.

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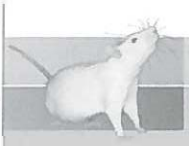

Curriculum vitae

Henriëtte Jolanda Hulshof was born on October 7th 1982 in Stadskanaal, The Netherlands. After attending high school at "Comenius College" in Stadskanaal, she completed the first year of a life sciences study in Emmen (HLO). She studied biology at the University of Groningen from 2000 until 2006 with a specialization in neurosciences based on three master research projects. Her first research project was performed in the Department of Molecular Neurobiology at the University of Groningen under supervision of Dr. Peter Meerlo and Dr. Karin van der Borght. She studied the effect of exercise on hippocampal neurogenesis in mice. For a second project she went to the Department of Neurobiology of Adaptive Processes at the Pierre and Marie Curie University in Paris, France, where she used a transgenic mouse model to study the postnatal development and organization of purkinje cells under supervision of Prof. Dr. Jean Mariani and Dr. Sonja Janmaat. During her final research project at the Department of Medical Physiology at the University of Groningen she studied the development of dopaminergic neurons from embryonic neural stem cells under the supervision of Dr. Sjef Copray and Dr. Veerakumar Balasubramaniyan. Soon after her graduation, she started her PhD project at the Departments of Molecular Neurobiology (University of Groningen) and Psychiatry (University Medical Center Groningen) under the supervision of Prof. Dr. Johan A. den Boer, Prof. Dr. Paul G.M. Luiten and Dr. Peter Meerlo, which ultimately resulted in the present thesis, titled "Early life influences, sex differences and stress vulnerability: The impact of maternal separation and sex on adult stress sensitivity in rats".

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